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Chapter 2

IN SITU PROTEIN CRYSTAL DIFFRACTION SCREENING

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ABSTRACT

The generation of high quality diffracting crystals remains an area that requires considerable person-power. Not only do crystals need to be produced and/or optimized, but such samples also have to be analyzed for their diffraction properties. While crystal shape and size are critical parameters controlling diffraction strength, diffraction screening remains the optimal manner to guide crystal growth optimization protocols. Mounting of the crystals to an appropriate sample support often requires transfer into a buffer significantly different from that the crystal is grown in, often resulting in a negative impact upon crystal morphology and diffraction properties. Modern synchrotron sources are increasingly using *in situ* exposure to X-rays to characterize protein crystals, with a growing number of structures being solved directly *in situ*, without recourse to manual handling of delicate samples. An automated approach requires crystal identification, positioning and collection of X-ray diffraction data for analysis. However, limitations are imposed by the variation in crystal size, morphology and space group. Here we review the availability and limitations of both commercial and synchrotron based infrastructures for *in situ* diffraction screening. We review the status of methods to establish the basic geometric features of crystals, the limitations currently inherent with *in situ* screening methods and we also describe our and other researchers efforts to overcome these limitations.

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INTRODUCTION

Structure solution using macromolecular crystallography (MX) remains the predominant method in use to obtain information on proteins at an atomic level of detail (87% of the deposited structures in the Protein Data Bank (Rose *et al.*, 2011) were determined using MX, as of January 2012). This structural information is subsequently used for a variety of purposes, including the elucidation of novel protein folds, assessing molecular interactions within complexes or the optimization of compounds in a drug discovery pipeline. While the objective of these experiments may differ, they all require data to be collected to the best possible resolution from any given sample. Modern synchrotron sources are highly evolved to perform optimal data collection experiments from any given sample. Such evolutions include:

- Methods to generate data collection strategies based on a comparison of diffraction and background (eg. BEST (Bourenkov & Popov, 2006))
- Screening of sub-regions of individual samples (Bowler *et al.*, 2010)
- Optimal partitioning of the X-ray dose across samples (Flot *et al.*, 2010)
- Rehydration (Russi *et al.*, 2011, Sanchez-Weatherby *et al.*, 2009) and/or cryo-annealing (Harp *et al.*, 1998, Yeh & Hol, 1998, Kriminski *et al.*, 2002) of samples
- Low dose X-ray tomography to identify optimal sample regions (Brockhauser *et al.*, 2008)

However, in these cases the quality of the initial sample remains the limiting factor. Simply put, an unoptimised sample is unlikely to generate optimal data.

Thus, a significant portion of any MX structure determination project is contingent upon the generation of the best possible samples. In many cases, sufficient data to address the specific question being investigated by the project (fold/ligand binding/etc.) may be achievable with suboptimal samples. However, more challenging projects that result in low-resolution diffracting crystals (eg. (Kato *et al.*, 2008, Tanaka *et al.*, 2009)) may be untenable unless significant efforts are made to optimize the sample prior to data collection.

A considerable amount of effort has already been made in the development of biochemical and biophysical methods to improve the chances of a successful crystallisation experiment (eg. Thermofluor assays (Nettleship *et al.*, 2008), additive screens (D'Arcy *et al.*, 2003), sample modification (Derewenda & Vekilov, 2006)). However, such methods are reviewed elsewhere and are not considered within this chapter. Nevertheless, the ultimate product of all of these approaches is another batch of crystals that need to be assessed for their diffraction quality. The classical approach for sample selection utilizes the biased opinion of an individual researcher. Samples would be selected for diffraction experiments based upon their size and morphology, qualities that imperfectly reflect the actual desired property for comparing their quality: diffractive strength.

Below we review classical and modern methods that are used to assess diffractive strength, with a particular emphasis on those that have features relevant to *in situ* diffraction screening.

ROOM TEMPERATURE VS CRYO-COOLING

Prior to the advent of cryo-cooling technologies (reviewed in (Garman & Schneider, 1997, Rodgers, 1997, Garman, 1999)), a manual mount of the crystals in a capillary containing the growth solution could be considered to be the optimal approach to assess diffraction quality. While this method minimizes the effects of buffer exchange on crystal diffraction, it does require a significant amount of expertise and manual handling. Such manual handling will always be an unquantifiable source of potential damage and could result in inaccuracies in the determined diffraction properties of any given sample. More recently, attempts have been made to improve this methodology with the introduction of systems such as the MicroRT™ X-ray capillaries (MiTeGen (Skrzypczak-Jankun *et al.*, 1996, Mac Sweeney & D'Arcy, 2003)), which integrates the handling advantages of loops commonly used for cryo-cooling within a hydrostatically sealed environment (Figure 1). This technique highlights one important consideration and advantage of *in situ* screening: the potentially adverse effects of cryopreservation buffers and the cryo-cooling process are avoided

Thus, the diffraction obtained from any particular sample is more closely representative of the true diffractive strength of the crystal. Conversely, the sample still requires manual handling with the associated and unquantifiable risks of damage and the introduction of systematic and non-systematic errors to the measured diffraction data.

While such room temperature methods have advantages; they also suffers from significant disadvantages. Firstly, the introduction of additional scattering material into the beam path (Figure 1) will generate an additional and undesirable background on the detector through stochastic (non-deterministic) scattering processes originating from non-periodic material present in the beam path. This background contribution mainly originates from Rayleigh scattering events and, while the expected distribution can be modeled, the individual scattering measured within any given unit area (ie. pixel) of a detector is non-deterministic and inherently unpredictable at low photon numbers.

Currently the additional contributions from Compton scattering (and other inelastic scattering events) can largely be ignored in the absence of materials that do not strongly absorb at the incident X-ray wavelength. However, the increased flux of future X-ray sources may require the background scattering from these events to be more rigorously addressed as these inelastic events may lead to increased background values measured on the detector.

While the increased background from the wall of the sealing material is to some extent quantifiable and/or reproducible, the background contribution from any buffer surrounding the sample is also highly challenging to quantify. This first limitation hinders a true comparison of diffractive strength, particularly if the sample diffraction limit falls with the area of the “water ring”. This common feature of protein diffraction experiments is an increased level of background between ~ 4 and 3 Å, due to the non-periodic arrangement of water molecules connected through a hydrogen bonding network.

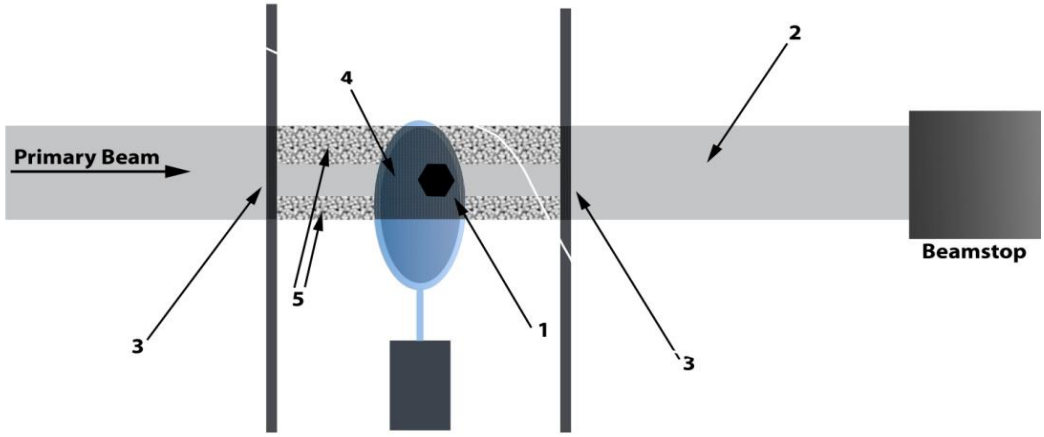

Figure 1. A schematic demonstrating the sources of background in a room temperature X-ray diffraction experiment. The diffraction received on the detector from the crystal (1) will have a background contribution from the air scatter produced by the primary beam (grey) as it traverses the experiment until it reaches the beamstop (2). Additional background contributions will be present from the capillary maintaining a hydrostatic environment around the crystal (3), as well as from any buffer and support materials surrounding the mounted crystal (4). Any beam that does not intersect with the crystal will generate no signal and will only contribute to background (5).

Secondly, the lack of cryo-cooling will severely limit the lifetime of the sample in the X-ray beam (Henderson, 1990). This second limitation makes any room-temperature approach unsuitable for the collection of complete high-resolution data sets from single crystals – although researchers are already improving methods to combine incomplete data sets from multiple samples (serial crystallography). Indeed, a number of structures have already been solved that required serial crystallographic data, with examples including the structure of the Blue Tongue Virus (Grimes *et al.*, 1998, Grimes *et al.*, 1995) and the free electron laser (FEL) generated structure of Photosystem I (Chapman *et al.*, 2011).

Third generation synchrotrons with narrow, intense x-ray beams are enabling data collection to be performed on ever-smaller crystals. Accurate 3D characterization of the crystal morphology and location has the potential to improve data collection strategies, particularly in the case of experiments in which radiation damage could be an issue. Radiation damage in crystals can be minimized using micro diffraction beamlines by moving the crystal center region to another part of the crystal before the diffraction data quality begins to deteriorate. Moukhametzianov et al. (2008) introduced an intentional misalignment to the crystal center position. This has the effect of the crystal rotating around the beam center position with unexposed crystal material being introduced in the beam path during data collection, thus minimizing contributions from already damaged regions of the crystal. The strategy of misaligning the crystal rotation center reduces radiation damage as different parts of the crystal are exposed. An alternative data collection strategy may involve the merging of diffraction data obtained from multi-crystals, however at the expense of additional data analysis complexity. (ie. collecting data simultaneously from multiple crystals within the sample crystallization cell). With sufficiently small crystals photoelectrons are able to escape from the vicinity of the crystal and the level of energy density tolerated by the crystal prior to damage is increased (Moukhametzianov *et al.*, 2008, Nave & Hill, 2005). Radiation damage

of disulphide bonds by X-rays has shown to be reduced by the use of free radical scavenging agents such as ascorbate and quinone (Nave & Garman, 2005, Southworth-Davies & Garman, 2007).

In summary, the decision to perform a room temperature experiment will impose certain restrictions and difficulties in data analysis and interpretation. In the optimal case, room temperature diffractive screening data can and should be used prior to the optimization of cryo-protective and cryo-cooling protocols before the collection of a complete data set is attempted.

DIFFRACTION STRENGTH METRICS

The discussion below makes continuous reference to "diffraction strength". However, no such concept is currently rigidly defined. Different crystallographers may choose:

- maximum resolution of observed diffraction,
- an arbitrary Rfactor cutoff on the observed data,
- an arbitrary I/σ(I) cutoff of the observed data,
- maximum diffraction spot intensities observed on any given image,
- apparent crystal mosaicity, particularly as it pertains to non-isotropic (streaky) spot shapes,
- diffraction spot shape,
- etc.

The ultimate goal of a diffraction experiment is the collection of a complete dataset to the highest achievable resolution and of quality that is sufficiently high to answer the question addressed by the experiment. We believe that an appropriate metric should be based upon the use of data collection strategy programs (eg. BEST (Bourenkov & Popov, 2006) and HKL-3000 (Minor *et al.*, 2006)) and algorithms that predict the longevity of a sample in the X-ray beam (eg. RADDOSE (Paithankar & Garman, 2010, Murray *et al.*, 2004)). Such algorithms allow a reasonable prediction of the final quality of a dataset collected with the X-ray dose appropriately portioned across the required rotation range. The individual experimenter can then choose a suitable metric for diffraction strength according to his or her personal preferences. Given that maximum likelihood approaches for model refinement (eg. REFMAC (Winn *et al.*, 2003, Winn *et al.*, 2001, Murshudov *et al.*, 1999) and PHENIX (Adams *et al.*, 2011)) can now make good use of weak data, one suitable metric could be the resolution at which the average I/σ(I) for the highest resolution reflection bin exceeds zero.

SOURCES OF BACKGROUND AND THEIR EFFECT ON DATA QUALITY

Any given X-ray source can be quantified in terms of the essential parameters that affect the final diffraction data quality (wavelength, flux, beam size, divergence and energy dispersion). Once these beam parameters are known, the diffraction intensities recorded on the detector from any given sample are a function of the beam characteristics, of the

experimental parameters (e.g. exposure time, diffraction geometry) and sample properties (volume of sample in the beam, diffraction strength). In this approximation the diffraction strength is a function of the long and short range ordering of the sample lattice as well as the asymmetric unit volume. While this diffraction strength parameter is difficult – if not impossible – to quantify without a full structure solution, the diffraction strength of any given sample is the parameter needed to guide crystallization optimization. However, it is possible in principle to rank a series of samples from crystallization optimization experiments if the beam parameters and crystal size are known. The crystals should belong to the same crystallographic space group for the ranking to be optimal.

As indicated above, all other non-crystalline material in the beam path (Figure 1) will also contribute to the scattering as measured on the detector. While this does not, in the first approximation, significantly affect the intensity of any given diffraction maxima as recorded on the detector, it will result in significant increases in the background recorded. The background increases not only in the regions surrounding the Bragg reflections, but it also increases at the peak location. In an example, two individual samples have identical size, morphology and diffraction strength, but a larger volume of buffer surrounds sample 2 (Figure 2). If the diffraction images of the two are compared, sample 1 will appear to be of better quality, as measured by the signal:noise ($I/\sigma(I)$) ratio of any given diffraction maxima. This situation can be further complicated when one realizes that the buffer components of any two samples in an optimization experiment may differ, with the attendent effect on background scattering from the solution, as well as upon the resulting sample size, morphology and diffraction strength. This effect is due to the inherent differences in scattering of the different atomic composition and/or concentration of the chemical components of the crystallization buffer and their associated X-ray scattering form factors. This can be of particular importance in the characterization of micro-crystals and weakly diffracting samples.

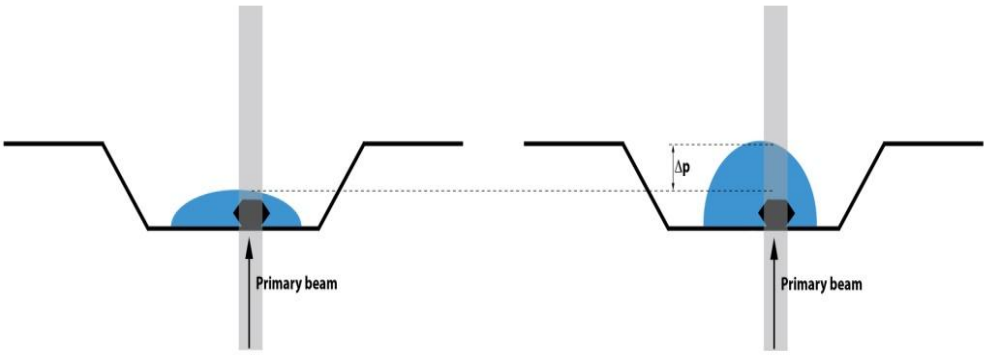

Figure 2. A schematic illustrating the effect of primary beam path in buffer. In the figure above both crystals are of identical diffraction properties. The additional path (Δp) traversed by the primary beam (grey) through the buffer surrounding the crystal will result in a significant increase in background on the detector. This will then result in an apparent decrease in the $I/\sigma(I)$ of the data collected.

While the background scatter from solution is stochastic, a number of data reduction and data collection strategy prediction software packages use fitted background functions to estimate the background contribution within any given reflection (eg. MOSFLM/SCALA (Leslie, 2006, Battye *et al.*, 2011, Evans, 2006), XDS/XSCALE (Kabsch, 2010), HKL3000 (Minor *et al.*, 2006), BEST (Bourenkov & Popov, 2006))

The use of these functions provide better integrated intensities, leading to more accurate I/σ(I) measurements for each recorded reflection and thus a more accurate estimate of the crystal diffraction quality.

In a simple approximation, the average background ($\langle I_b \rangle$) for a particular area of the detector and the average background accuracy (ie. standard deviation) ($\langle \sigma(I_b) \rangle$) can be calculated from the intensities (I_b) of the N background (ie. non-Bragg region) pixels of an image using the following equations.

$$\langle I_b \rangle = \frac{1}{N} \sum_{i=1}^{N} I_{b,i}$$

$$\langle \sigma(I_b) \rangle = \sqrt{\frac{\langle I_b \rangle}{N}}$$

This approximation is correct provided that the background is flat (all I_b values are drawn from the same distribution) and their values are high, so that Poisson's Law holds and the error in their values are uncorrelated.

The minor limitation with the use of fitted functions is that the position, number and value of the detector pixels used for fitting will limit their accuracy for any specific reflection; N ideally should be large. In addition, weaker reflections will be inherently less accurately measured, simply as a result of counting statistics, implying an associated loss in accuracy in the characterization of weakly diffracting samples. Weaker intensities have lower standard deviation following Poisson's Law and, as the background intensity is subtracted from the peak intensity, weaker peak intensities will lead to larger errors in the integrated reflection intensities. This drawback can be somewhat overcome by simply choosing the strongest (eg. I/σ(I) > 10) reflections for analysis, although the associated reduction in the number of reflections used for sample diffraction strength comparison will also affect the quality and accuracy of any such comparison.

Additional sources of background can also include the air path that is traversed by the primary beam as well as any material within the crystallization equipment. To an extent, these contributions can be neglected, as generally the contribution will be highly reproducible (eg. crystal to beamstop distances and beam sizes; Figure 1) Additionally, steps have already been made to optimize the plastics used for *in situ* diffraction screening (le Maire *et al.*, 2011), (Kisselman *et al.*, 2011), (Minor *et al.*, 2006) TOPAZ® Diffraction Capable Chip (Fluidigm (Hansen *et al.*, 2002)), etc.). This consideration will be discussed further below.

In summary, algorithm-based predictions of background can improve the accuracy of measurement of reflection I/σ(I) values, although more weakly diffracting samples will

remain inherently more problematic. A more significant hindrance to accurate *in situ* screening experiments is the lack of information on crystal size and morphology.

CRYSTAL MORPHOLOGY AND SIZE

The volume of a sample exposed to the X-ray beam is directly proportional to the total scattering of any given diffraction maxima. Thus, any robust comparison between the diffraction strengths of crystals requires knowledge of the sample dimensions, morphology and orientation during exposure. In the comparison shown in Figure 3 non-identical diffraction patterns are obtained from samples of identical diffraction strength, purely as a result of the reduction in volume of the sample within the beam. The approach of using 1μm sized beams (Bowler *et al.*, 2010), such that the beam size is smaller than the geometric (ie. scattering) cross-section of the crystal normal to the beam path will partially address this issue. However, the varying depth of the different samples within the beam ("Δd", Figure 3) will still contribute to the overall scattering volume and the resulting diffraction.

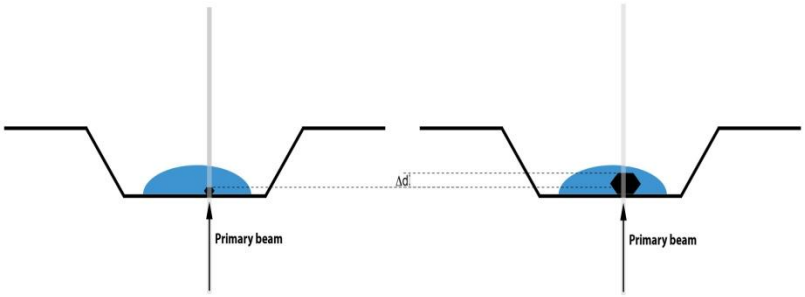

Figure 3. A schematic representation of the effects of crystal volume on diffraction intensity. The crystals in the images above are of identical diffraction properties. However, the volume illuminated by the primary beam is significantly different. This will result in a significant differences in diffraction intensity recorded between the two examples.

A further complication arises from the fact that crystals are commonly found in a number of differing morphologies as screening conditions approach optimal crystallization conditions. In addition to variations in overall size, crystals may appear as single plates and needles (Figure 4). This variation in crystal shape should be taken into account before any attempt to assess the diffraction strength is made. However, no automated approaches to classify shape and/or size exist in macromolecular crystallography, although our own and other researchers work on crystal recognition based on fluorescence (Forsythe *et al.*, 2006, Groves *et al.*, 2007, Watts *et al.*, 2010) and Ultraviolet (UV) absorption (Judge *et al.*, 2005, Madden *et al.*, 2011) holds the potential to make a computational assessment of crystal shape. Perhaps the use of low dose X-ray tomography (Brockhauser *et al.*, 2008) offers the best potential for the future. A single low-dose image of the entire crystallization experiment could be recorded prior to *in situ* screening and reconstructed into a density map that would indicate the volumes of the crystals. Such densities could then be used as a map to guide micron-sized beam screening,

with the resulting diffraction intensities and measured diffractive volumes used to calculate diffraction strength.

Finally, the varying morphologies of crystals produced during crystallization optimization may also include conditions in which clusters of plates and/or needles are found. This would introduce a further complication, as multiple diffracting objects would be simultaneous screened. One potential approach to counteract this situation would be to follow the suggestions in which cryo-mounted crystals could be ablated into pre-specified shapes using UV- or Infrared (IR) sources (Kitano *et al.*, 2005, Kashii *et al.*, 2006). In this approach sections could be cut of predefined size from each crystal to be screened *in situ*. This methodology could be of use as a potential mechanism to separate clusters of crystals, without the need to disturb the crystallization experiment, although the effects of local heating upon the crystals due to the ablation process would have to be thoroughly assessed and monitored. Methods would also need to be developed that can accurately and automatically distinguish crystal clusters from single crystals.

Traditional optical based crystallization detection methods have difficulty in determining the difference between amorphous precipitate particles and micro crystals that can in part be attributed to low spatial resolutions achieved on the whole drop imaging, high level of jpeg compression of images and the transparent nature of protein crystals. Bingel-Erlenmeyer (2011) using the CrystalQuickX plate for *in situ* data collection found that microcrystals at high enough concentrations gave 'powder like' ring diffraction patterns. The use of *in situ* diffraction enables microcrystals to be differentiated from precipitate and therefore has the potential to offer important additional information concerning the crystallization growth phase process for each crystallization trial.

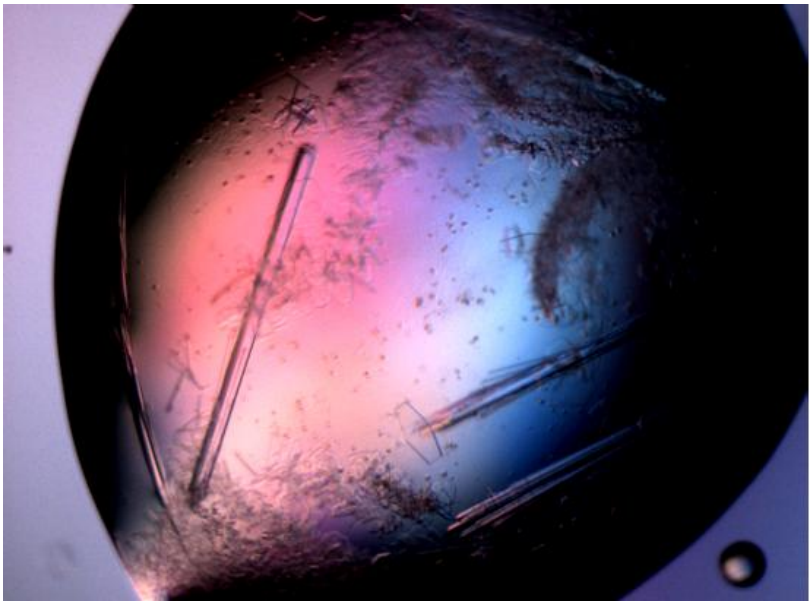

Figure 4. An example of different crystal morphologies found within the same crystallization condition (Hatzopoulos *et al.*, 2008). Plates and needles morphologies are present under the same crystallization condition.

CRYSTALLISATION PLATES FOR *IN SITU* DIFFRACTION

The Greiner BioOne CrystalQuick X™ crystallization plates have been developed in conjunction with the FIP beamline at ESRF Grenoble for use with the G-Rob 2D automated crystal screening system (Bingel-Erlenmeyer 2011). The traditional SBS format crystallization plate has been redesigned to enable an oscillation range of 80° and by using plastic material that is 3 times thinner for the sitting drop crystallisation area a reduction in the level of background X-ray absorption is achieved (Figure 5). Other groups have investigated the used of microfluidic chips (eg. (Dhouib *et al.*, 2009, Kisselman *et al.*, 2011)) or capillary plates (eg. CrystalHarp™, Molecular Dimensions Ltd.) for *in situ* diffraction analysis. However, the relatively high level of background scatter remains an issue with cyclo-olefin- copolymer (COC) based crystallization supports. Microcapillary approaches (as exemplified by CrystalHarp™) are composed of quartz and possess a significantly lower background (Figure 5.).

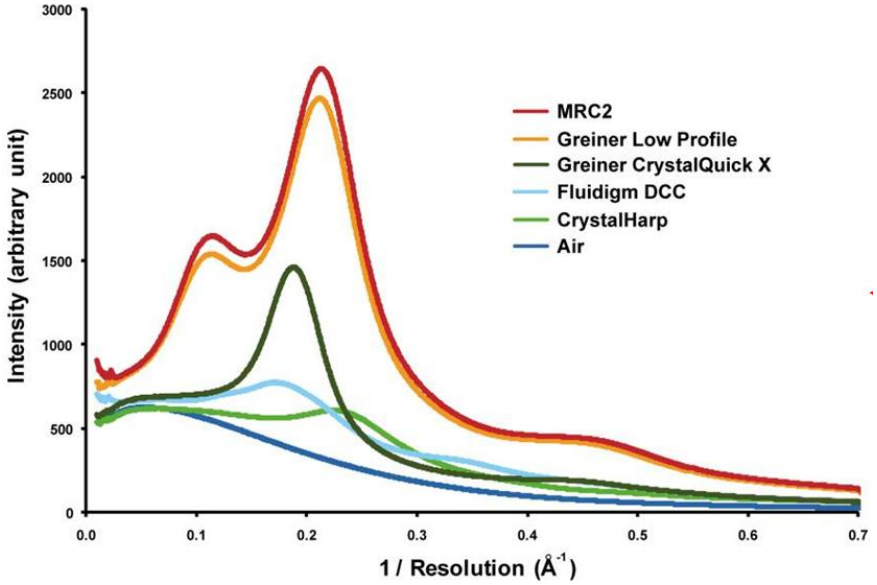

Figure 5. Levels of X-ray background measured for different crystallization plates (reproduced with permission from Bingel-Erlenmeyer (2011)). The best performing plates are the Fluidigm Diffraction Capable Chip and Crystal Harp that achieve background absorbance's levels close to that of air alone.

Sandwich design crystallization plates are also being developed at EMBL Hamburg (Watts *et al*., manuscript in preparation) that enable crystals to be grown and diffraction data collected *in situ* without the requirement for invasive handling. The crystals are grown within micro batch cells that use outer layers of 4 μm thick X-ray transmissive material in the X-ray beam path. This has the effect of reducing background absorption originating from the crystallization plate to levels comparable with that of ambient air, with the exception of faint rings at 2.2 Å and 2.7Å (Figure 6). The thin plate profile (~1 mm) enables a wide data collection angle thus facilitating crystal indexing/ scaling between multiple crystals. Future work will address creating the convenience of SBS format sitting drop crystallization plate by

incorporating the thin film technology of the sandwich plates into the crystallization drop well.

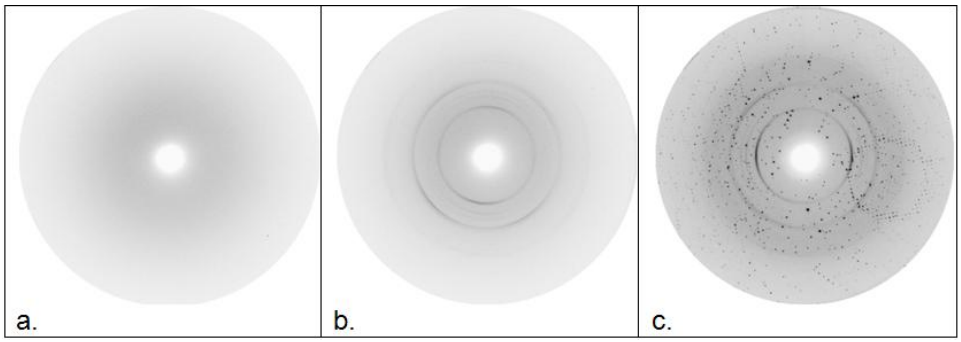

Figure 6. Diffraction patterns obtained on EMBL X13 beamline using a prototype *in situ* compatible support. a) air (control) b) using EMBL prototype sandwich plate without crystal c) EMBL sandwich plate including diffraction from lysozyme crystal. The thin 4μm film material within the beam path limits the presence of background to 2 faint rings.

CURRENTLY AVAILABLE *IN SITU* SCREENING POSSIBILITIES

Although a great deal of development is still needed to optimize and automate accurate *in situ* screening there are already a number of possibilities that allow researchers to perform *in situ* analysis of their crystals. A growing list of synchrotron beamlines now allows users to characterize the diffraction from standard 96-well plate crystallization trays. For an up-to-date list the reader is referred to www.lightsources.org. The limitations reviewed above can, to a certain extent, be addressed through the expertise of the user in choosing beam size, sample choice and assessing the resulting diffraction image. Such experiments can also be readily performed remotely, as the sample throughput and quantity of data generated is much lower than that of a 3rd generation source running in "data collection" mode (up to several frames per second). Additional time is consumed in bringing individual samples to the beam centre – requiring precise positioning of the crystallization plate.

An increasing number of macromolecular crystallographic beamlines have associated crystallization robotics nearby (eg. (Mueller-Dieckmann, 2006)), allowing for the potential to ship proteins for crystallization on-site, rather than ship the results of delicate crystallization experiments. This significantly reduces the risk of damage in transit with the attendant increase in confidence in the results. These additional time requirements, while reducing the potential throughput and efficiency of any particular beamline, are arguably offset by the significant improvements in the time and cost associated with any structure determination project. This nominally less efficient use of beamtime will result in great savings in the person power and time required to obtain a useful structure.

Any *in situ* screening performed on a 3rd generation source is likely to be limited to a single exposure, due to the effects of radiation damage on samples that are not cryo-cooled (Henderson, 1990). Software that estimates background contribution, such as BEST (Bourenkov & Popov, 2006), can estimate diffraction strength from a low dose experiment. Thus, one can screen crystals at a low X-ray dose and then estimate the limit from

extrapolation. This approach would be of more use in experiments on optimized crystals. In experiments that are designed to generate data for subsequent optimization of crystal growth it is more reasonable to estimate the diffractive strength based on a high X-ray dose experiment, as these crystals are used to guide the optimization process rather than for final data collection.

A commercial solution also exists (PXScanner™ (Agilent Technologies)), in which a rotating anode is used as the X-ray source. While highly convenient, this system requires exposure times of 5-10 minutes for reasonably diffracting crystals that may be even longer if the *in situ* samples to be screened are of a few microns in size. However, such a system will rapidly differentiate between protein and salt crystals and allows immediate testing, rather than waiting for sample shipping and/or beamtime scheduling. The approaches used in BEST (Bourenkov & Popov, 2006) for estimating diffraction strength at a low dose may be adapted for these cases, although the different beam properties should be taken into account.

Finally, while all the advantages and limitations discussed above may lead to future optimization of *in situ* screening, they also point to a clear need for expertise in data collection and analysis within each laboratory that generates protein crystals for structure determination. No amount of automation will address all potential difficulties and an experienced eye will always be able to improve the interpretation of data.

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