

Diamond Light Source

Outline Proposal for a Phase III beamline

A sub-micron capable varifocus MX beamline with a side-station for *in-situ* diffraction

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1. Executive Summary

We propose to complement the existing set of state-of-the-art MX beamlines at Diamond with a next generation macromolecular crystallography (MX) beamline. This will harness advances in microfocus beamline design and recent breakthroughs in the *in situ* diffraction analysis of crystals, to provide a beamline which will deliver major benefits to the entire UK MX community, both academic and industrial, for whom the generation of diffraction capable crystals is the major bottleneck on their productivity. In particular the proposal will enable the study of challenging biological systems that are beyond the capabilities of the current set of beamlines and will also pioneer the next phase of automation, shortening the path from crystal screening to structure solution.

The proposal encompasses two highly complementary end stations which address the fundamental problem of dealing with micron sized crystals: (1) a high flux density, submicron varifocus beamline that can address the challenge of ever smaller crystal samples and/or identify “sweet spots” in larger crystals; (2) a side station that will significantly increase the scope of macromolecular crystallography by directly characterising crystallisation trials *in situ*, permitting the automatic characterization of crystal diffraction properties, and in some/many cases allow data acquisition without the need to cryoprotect and manually mount crystals. To enable the effective use of these beams existing automation will be extended to (i) include tomographic-enabled matching of beam to crystal and (ii) include *in-situ* analysis seamlessly within existing crystallisation pipelines.

2. Overview

We propose a facility which will advance the capabilities of MX to rapidly characterise and collect high quality data from the most challenging crystals. This will enable the following areas of science in particular: (i) membrane protein crystallography, (ii) large macromolecular complexes, (iii) virus crystallography.

To achieve this we address the following specific technical challenges: (i) early stage crystal hit identification and optimization, (ii) crystal manipulation, handling, harvesting and freezing, (iii) fully integrated and optimised crystallization, data collection and structure solution.

We propose two experimental stations. The first is a **tuneable submicron focus line** (6.5 – 30 keV) with dynamic focusing. The beam size is continuously and independently variable between 0.5 and 10 μm in each linear dimension, to allow the precise matching of beam size and shape to very small crystals, thus optimising the signal-to-noise ratio. Additionally, it will allow larger inhomogeneous crystals to be scanned for well diffracting regions. The high energy regime (above 20 KeV) will exploit the photoelectron escape thereby reducing radiation damage (1, 2). A fixed beamsize of 300x300 μm^2 will also be available to illuminate the whole sample on demand for microtomography. The unique combination, of microtomography and microdiffraction will permit rapid location and shape determination of otherwise hard to identify or invisible crystals (e.g. micron sized crystals or crystals embedded in opaque lipid cubic phase - see *Figure 1*) and enable their immediate diffraction analysis.

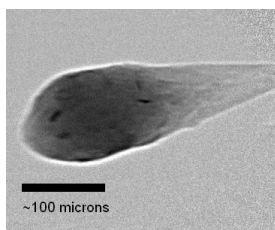


Figure 1 Microcrystals of bacteriorhodopsin within lipid cubic phase material made visible by Xray microtomography (measured on 103 with $\lambda=2.5 \text{ \AA}$).

This capability is complemented by the side station, which provides for **high throughput *in-situ* analysis**, with particular potential for crystals of complex systems, for instance membrane proteins, where apparently nicely shaped “crystals” frequently show little or no diffraction. Here, users can automatically characterize crystals in a variety of crystallisation setups for diffraction properties, providing rapid feedback for subsequent crystal

optimisation experiments, thereby saving considerable time and money. Initial crystal identification will use vis/uv imaging, and low-dose X-ray tomographic stereo imaging will be investigated. Moreover, the endstation will be optimised for the collection of complete data sets at room-temperature (or reduced temperature) which may require a large number of crystals due to radiation damage. The provision of this optimised sample environment is a key deliverable to enable the full exploitation of the *in-situ* technique. In summary, the seamless combination of *in situ* diffraction

with multi-crystal data collection will significantly extend the experimental envelope for the type of sample and modes of data acquisition for the UK structural biology community.

3. Scientific Case

MX remains the most important analytical technique for understanding biological structure-function relationships at the molecular level (26 Nobel prizes to date). Over the last three decades MX has not only benefitted from, but has also driven investments in synchrotron facilities and contributed immensely to the development of beamline optics, end-station, detector technology and beamline automation. In addition to its contributions to fundamental science MX is central to the therapeutic pipelines of the pharmaceutical and biotech sectors, making it the dominant single industrial application of synchrotron radiation (SR).

Combined with major progress in molecular biology over the same period, advances in SR-MX have provided many non-expert users an easy entree into MX: in the ever-expanding community experiments that were cutting edge crystallographic challenges 20 years ago are now routinely performed in minutes. During this period the crystallographic technique has, in a sense, been “industrialised”, in part through structural genomics/proteomics initiatives, increasing the productivity and accessibility of beamlines. It is these developments that have enabled crystallographic data to be an integral part of modern day drug discovery and development by both large pharmaceutical and smaller biotech companies, since the crystallographic analysis can rapidly respond to, and inform, the efforts of medicinal chemists. The remarkable advances show no sign of abating and Diamond plans to maintain its existing beamlines at the cutting edge, through continued investment in the development. However, there are now opportunities of more radically exploiting the opportunities of microbeams and establishing a highly integrated crystal growth/optimisation loop which will advance the boundaries of practical crystallography and continue to empower the broader life science community.

A vital consequence is that the complexity of crystallographic problems has multiplied - many groups are tackling large multi-subunit complexes which function as macromolecular machines to control processes such as cell division, cell-communication and signalling, chromatin remodelling and gene expression. To give a single example - the anaphase promoting complex (APC) regulates cell cycle transitions by mediating the destruction of certain proteins at specific phases of the cell cycle. The APC is composed of 18 subunits (from 14 different proteins) with a mass of 1.2 MDa. The APC has very low natural abundance, but advances in multigene expression in insect cells has allowed the *in vitro* reconstitution of mg quantities of a pure, active, complex suitable for crystallisation screening (3). When crystals are obtained, access to a tuneable microfocus beam-line and in-plate crystal screening would significantly facilitate structure determination. This has been exemplified by the recent ground-breaking elucidation of GPCR structures for which microfocus beamlines have been a pre-requisite (4). The future possibilities are exemplified by recent results from I24 which demonstrate that many megadalton virus particles can now be analysed routinely using the combination of optimised microfocus capability and plate screening. The challenges of visualising complex macromolecular machines are the frontier of structural biology that drives this proposal. To this end the current proposal addresses the two major issues in modern MX: (i) obtaining and identifying suitable crystals for diffraction experiments and (ii) collecting diffraction data from samples that would otherwise be discarded because of size, inhomogeneity or diffraction quality. The facility will provide a unique opportunity for all UK structural biologists to gain advantage from a two-pronged approach to their hardest scientific problems: the use of X-ray diffraction to directly probe the quality of their initial crystal samples and the use of microbeams for extracting useable data from the most problematic of samples. Diamond already has a leading microbeam MX beamline, I24. This beamline is unable to meet the existing demand for either plate or conventional microbeam applications. The current proposal aims to meet the increasing demands from the UK community by putting in-place a station which will build upon I24 experience but will provide capabilities beyond any existing MX beamline, for instance combining microbeam diffraction at high X-ray energies and X-ray tomography at low energies.

The second prong of the attack derives from the observation that high throughput (HTP) approaches to protein production and crystallization have demonstrated that for many projects initial crystal hits and in some cases diffraction quality crystals can be obtained by automated means. Current methodology still depends on manual harvesting and freezing of crystals, requiring optimization of cryoconditions, in preparation for synchrotron beam time. Recently it has been demonstrated that multiple early stage crystal hits from HTP crystallization pipelines can be used to record diffraction data *in situ* of sufficient quality to yield structure solution by molecular replacement for proteins, whilst new virus structures have been obtained at high resolution from as little as 20 micrograms of material using *in situ* diffraction. These results demonstrate the potential for an effective pipeline running from gene to structure.

Submicron varifocus station

Crystal size remains the limiting factor in many MX projects. Although recent advances in beam line technology mean that crystals with linear dimensions of 50 μm are often sufficient for data collection, only a few beam lines have produced useful data for crystals smaller than 10 μm in linear dimensions (most notably ID13 and ID23-2 at the ESRF, X06SA at the Swiss Light Source and I24 at Diamond (5)). Current challenges include membrane proteins, some multi-component complexes, and fibril forming proteins (6-8). Crystal size may be limited by molecular inhomogeneity and flexibility, the presence of inclusion bodies or by the quantity of protein available. The difficulties associated with obtaining sufficient pure protein material for many of these challenges has driven the use of nanodrop crystallisation facilities with microfluidic devices becoming available. The inevitable consequence is smaller and smaller crystals.

Crystal aspect ratio: optimisation of the signal-to-noise ratio is crucial for micron size crystals and relies on minimising background scatter, requiring a close match of beam size to crystal. Many macromolecular crystals are thin plates that present rapidly varying aspect ratios as the crystal is rotated during data collection. An X-ray beam line that can provide a variable aspect ratio beam provides the best chance of recording complete high quality data in such cases (9, 10).

Macromolecular crystals are not uniform in nature – they grow with imperfections and varying degrees of disorder. Multicomponent complexes often show a high degree of inhomogeneity, in particular if extracted and purified from natural sources. Moreover, the mechanical process of mounting, exposure to air and sample dehydration, cryoprotection and flash freezing affects crystal integrity. The micro-focus station proposed here will be able to rapidly scan larger crystals in three dimensions to find the optimal areas for data collection.

Radiation damage is the limiting factor in many challenging biological crystallographic investigations. Investigation of small crystals results in exposure of small diffracting volumes that then require high dose levels to achieve a reasonable signal-to-noise ratio. Conversely, data collection on micro-crystals offers the potential of avoiding secondary damage since the excited photoelectrons have an average path length of a few microns and escape the sample (1) A recent study highlighted a significant reduction in radiation damage utilising sub-micron beams (2). The path length increases with increasing X-ray energy, dictating the 30keV capability proposed.

In-situ side station

Currently most SR MX determinations rely on cryogenically protected frozen crystals to minimise radiation damage. However, the great success of cryo-data collection has overshadowed two ever present challenges that are addressed by the *in situ* HTP side station proposed here: Crystallisation is often a very long and iterative process where initial *promising* conditions are optimised until diffraction quality crystals are obtained. But, crystallisation is in almost all cases separated from the

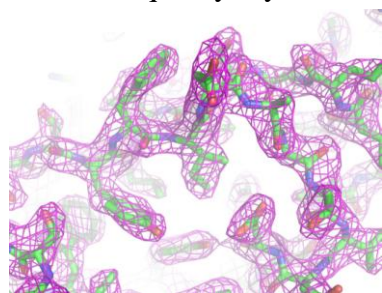


Figure 2 Electron density from MR phased human protein using data measured from 50 initial hit crystals in 3 drops from Greiner crystallization plates. Collaboration with I24, Ray Owens (OPPF) and Ann Morgan (Leeds IMM).

diffraction experiment. Crystals are usually optimised on the basis of their appearance, due to the impracticality of sending every potential sample to the synchrotron.

The need for *in situ* diffraction in crystallisation trays has already been recognised as exemplified by the development at Diamond and other synchrotrons (I24 at Diamond, X06DA at the Swiss Light Source, FIP at ESRF) and commercial solutions (Agilent Diffraction Scanner). This capability provides a competitive edge for membrane proteins, especially in the highly viscous lipid cubic phase, where harvesting is very tricky. *In situ* measurements provide a non-invasive assessment of diffraction quality, allowing different crystal morphologies to be explored without the influence of freezing. By providing suitable goniometry the proposed beamline will enable the collection of complete datasets, obviating the need for harvesting many samples (which routinely leads to sample loss or damage). Each dataset would typically require a large number of crystals, because of rapid decay at room temperature; conversely, at room temperature isomorphism between crystals is typically very good and mosaic spread is low (11). As noted above experiments on the microfocus beamline I24 with both protein (Figure 2) and virus crystals, have shown that high quality structures can be obtained from a single 96-well crystallisation tray using this approach. Transporting crystallisation plates to beamlines has been common practice for some groups for many years and is underpinned by robust procedures and protocols. An alternative route is to use dedicated crystallisation facilities which can be accessed remotely. Such facilities are already available on site at Diamond (and elsewhere, e.g SLS, PetraIII and ESRF). The present proposal would integrate with existing on-site crystallisation facilities to the advantage of the wider life science community.

Outline Specification for submicron varifocus station

Endstation	Sub microfocus	In-situ
Energy range (keV)	5 – 30	6 - 12.7
Beamsize (µm)	0.5 – 10 (300)*	5 – 50**
Flux (phs/s)	> 10 ¹³ in a 1 x 1 µm spot	at least 10 ¹²
Goniostat	Nanospindle rotation axis with a sphere of confusion of 100nm	Versatile goniometer with large translations (positional accuracy in the 0.5 – 1 µm range) for accessing all positions in a SBS standard plate while permitting high angular rotation around any selected crystal position (±30°)
Sample handling	Automounter with high capacity and fast cycle time (<30s)	Two storage vaults, each of ~1000 plate capacity, including vis/uv imaging and operating at different temperature (eg 20 and 4 °C)
Sample environment	Cryo-cooled, humidity controlled and in-line spectroscopy	Automated plate transfer system and collocated plate hotels and imaging systems at 4-25 °C
Sample visualization	On axis viewing	Integrated beamline plate-imaging software including advanced remote inspection, experiment planning and scheduling
Tomography	Integrated into on-axis viewer	If appropriate X-ray tomography of drops
Detector	>6 Megapixel High frame rate (>50Hz) area detector with a 1-3 millisecond readout time optimized for use over the stated energy range.	Fast frame rate detector (at least 100 Hz) with an active area of >300 mm and a 100hz frame rate with 1-3 millisecond readout time

*The focused beam size at the sample will be continuously variable independently in the horizontal and vertical directions from 0.5 -10 µm. In addition the beamline optical design will allow the user to change to a fixed large beam configuration of at least 300 µm for tomographic applications in near real time. ** The focused beam size at the sample will be continuously variable independently in the horizontal and vertical directions from 5 -50 µm.

4. Community

The UK MX community remains perhaps the strongest in Europe, numbering well over 100 PI's and its research is of strategic relevance to the Wellcome Trust, the Medical Research Council, Cancer Research UK and the Biology and Biotechnology Research Council. In addition, access to cutting-edge MX is of key importance to the Pharma and Biotech industries for which structure based drug discovery is an integral part of the drug discovery process.

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