The Development of an Optofluidic Evanescent Field Sample Trapping and Loading Solution for Time Resolved Protein Crystallography Experiments

A. J. Diaz*, P. Docker+, M. R. Sparkes*, J. Kay+, D. Stuart+, J. Beale+, D. Axford+, W. O'Neil*, B. Cordovez^

+ Diamond Light Source, Didcot, Oxfordshire, UK
* University of Cambridge, Cambridgeshire, UK
^ Optofluidic Philadelphia

ABSTRACT

X-ray crystallography is the leading technique for determining the atomic structure of biological molecules. The developers of this technique have maintained this position by continuously pushing the hardware and software boundaries such that data-collection from micron sized crystals are now possible. However, to break through the micron barrier will require a paradigm shift. Currently, the majority of samples are mounted on cryo-cooled pins. Mounting and aligning a micron and nano metre sized crystal to an X-ray incident beam is beyond the limits of electromechanical goniometers whose inherent limitations such as movement backlash impair practicable manipulation and alignment on this scale.

Currently proposed alternatives have had varying degrees of success, but have yet to unlock the coveted submicron sample-loading domain. We propose a novel sample loading methodology, which combines elements from microfluidics and optical tweezing. The device is an optofluidic chip and nano-tweezers system that traps samples via evanescent fields emitted from silicon nitride waveguides transmitting a 1064 nm laser beam. Crystals can be aligned in solution, negating the need for pins and cryo-cooling systems.

This paper presents a further development of the system's application. The highly beneficial implementation of microfluidics was further exploited by upgrading the system to allow for the control of one or more microfluidic ports and flow streams. This not only improved the tweezing process but it also created the ability to introduce multiple samples and reagents. Combining these during both the sample feeding and tweezing stages has allowed for the possibility of using the x-ray crystallographic method to capture ongoing reactions as they occur. These 'time resolved' nano crystal experiments are currently beyond the capabilities of the crystallographic method but remain the ultimate quest of many structural biology projects as they could reveal the complex structural changes of enzymes as they react in real time with their substrates. The optofluidic chip is currently being used to explore the mechanism of the beta-lactamases; several families of proteins which catalyse the hydrolysis of beta-lactam antibiotics. These proteins are ideal for this task, not only because the biological question is exceptionally relevant in a post-antibiotic world **[1]**, but also several substrates undergo a colorimetric change during catalysis so the X-ray diffraction data can be obtained whilst monitoring the activity of the enzyme. These optofluidic chips have potential application for both synchrotrons light sources and X-FEL beamlines.

Keywords: X FEL, Sample Delivery, Time Resolved, Protein Crystallography, Optical Tweezing, Evanescent Field

1 INTRODUCTION

Within the last decade, X-ray crystallography has become the primary technique for determining complex structures in the biomedical and chemical engineering fields of research and manufacture, superseding NMR and 3D modelling. It is particularly effective for the characterization of protein structures. Such popularity has driven the improvement of both synchrotrons and x-ray free electron lasers (XFELs) hardware in an attempt to improve the quality of the measured data, resulting in technological leaps in terms of X-ray generation, detection and post experimental data processing. However, sample loading still falls behind in comparison and presents a new and exciting challenge.

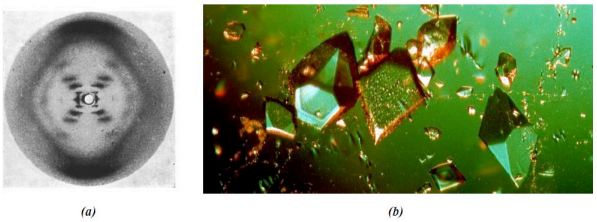

Figure 1. X-ray diffraction pattern of DNA (a) and protein crystals suspended in their mother liquor (b).

Currently, the majority of synchrotron beamlines around the world use either electro-mechanical goniometers or robotic arms to load micron dimensional sized protein crystals into the path of high power x-rays. Although this was

satisfactory in the early years of x-ray crystallography, the rapid development of powerful synchrotron light sources, more sensitive detectors and improved modelling software have swiftly outstripped the capacity for these current loading techniques, bottlenecking progress for this technology, particularly with an ever-growing interest in submicron crystal diffraction. The system described in this work is an exciting new approach that addresses these issues by automatic crystal delivery and operating at room temperature, which in turn lends itself to time resolved studies. For the first time a handling system that can isolate and hold submicron crystals and expose them to substrate to initiate a reaction is described. The application in this case is the study of Beta Lactamases, a protein responsible for making certain bacteria antibiotic tolerant.

2 THE BIOLOGY

A significant barrier in conducting these experiments currently is co-ordinating the presentation of the crystal, the mixing of the substrate with the crystals and then illuminating the crystal with X-rays. In this system, we propose to restrain the crystals with the optical traps and introduce the substrate solution to the crystals prior to X-ray radiation. We are currently using this system to explore the mechanism of the beta-lactamases; several families of proteins which catalyse the hydrolysis of beta-lactam antibiotics reducing the effectiveness of antibiotic treatment.

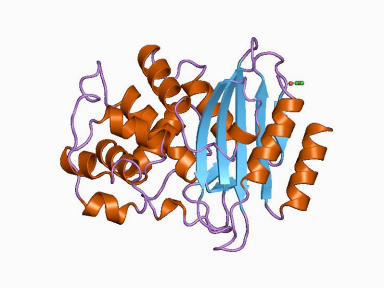

Figure 2. Protein structure of Streptomyces Albus Beta-Lactamase.

These proteins are ideal for this task, not only because the biological question is exceptionally relevant in a post-antibiotic world, but also several substrates undergo a colorimetric change during catalysis so the X-ray diffraction data can be obtained whilst monitoring the activity of the enzyme.

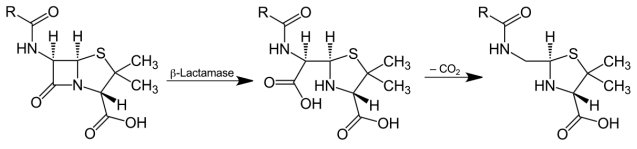

Figure 3. Action of β-lactamase and decarboxylation of the intermediate.

The same colour change can also be used to study the efficiency of the mixing taking place. The technical challenge this work focuses on the system's capacity to trap crystals beyond the micron scale and into the nano domain. This is of great interest, as smaller protein crystals allow for quicker substrate diffusion.

3 OPTICAL TRAPPING

The optofluidic chip is the core of this project. Its semi-conductor nature means that it can be easily manufactured, using modern and well defined industrialised processes. This gives it the potential to be a relatively cheap and mass manufactured alternative to other solutions.

The optofluidic trapping functions as follows: A 1064nm laser is transmitted through the silicon nitride waveguide, under total internal reflection. Transported through the microfluidic channel, the crystals will pass over the waveguide, which perpendicularly traverses the microfluidic channel. As the particles enter the evanescent field, they become trapped via dipole Rayleigh scattering. The trapping potential is a function of the input power from the Nanotweezer unit laser, along with coupling losses, and the forces applied by the microfluidic flow on the particle. These parameters have been characterised and balanced for each individual sample type.

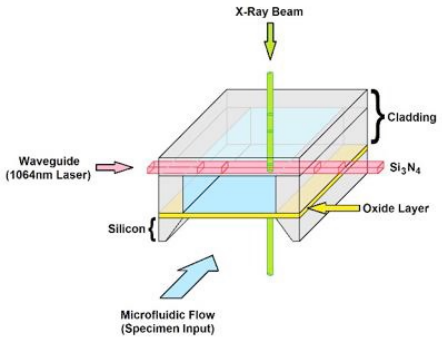

Figure 4. Diagram demonstrating the optofluidic chip architecture.

Samples are brought to the trapping area via microfluidic delivery, which offers the crucial advantage of keeping samples (specifically crystallised proteins) within their native mother liquor, thus eliminating the requirement for cryogenic cooling.

The trapped particles can be released in one of two ways: The first is to switch off the laser, disabling the trapping fields emanating from the waveguide and the other is to cause a sudden burst in the flow rate, which in turn will cause a stronger lateral force on the particles, and overwhelm them out of the trapping fields.

The optofluidics chip is 10mm by 10mm and is manufactured via semiconductor processing. It has a structure composed of three layers: silicon base; an oxide layer, on to which three silicon nitride (Si3N4) waveguides have been deposited; and a cladding layer. At the centre of the chip, a microfluidic channel has been etched, ending at either end in microfluidic I/O ports, which connect to the fluidic control system.

Traversing the microfluidic channel are the silicon nitride waveguides which measure 600 nm across and are 200 nm thick (see figure 5) and have 180nm holes evenly spaced along their length which are the sites of trapping due to concentrated effervescent fields. These chips are different from the standard chips as they have also been back etched through the silicon layer to eliminate X ray beam attenuation when carrying out transmission measurements. The micro fluidic channel is sealed using a Kapton window.

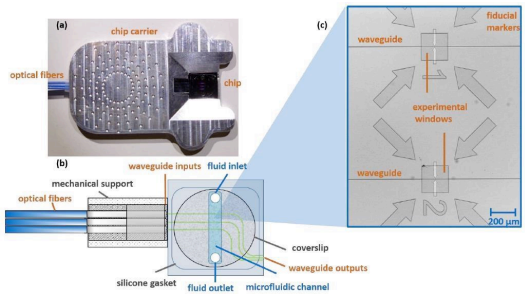

Figure 5. Photograph and diagrams representing the optofluidic chip and its internal structure.

The waveguides are coupled to the optical fibres in the fabrication lab and are held in place by an aluminium chip carrier, which secures both the chip and the pigtail launcher delivery in place, whilst also providing a magnetic anchoring solution. By using the waveguides to trap the crystals samples, the flow rate can be manipulated to introduce reagents over the crystals to initiate the reaction.

4 CURRENT SYSTEMS & LIQUID JETS

The majority of the nano crystals data collection involves liquid jets as a delivery system. By creating a high-pressure sheath around a central channel liquid jets of droplets containing crystals can be ejected and presented to the X-ray Beam.

Typical flow rates of sample are anywhere between 10 – 20 microliter/minute and the jet diameter typically about 6 µm. When the liquid jet is running, the vacuum in the experimental chamber is < $3x10^{-5}$ Torr. However although the tip that develops the liquid jet is small the infrastructure to support the jet is significant (figure 8)

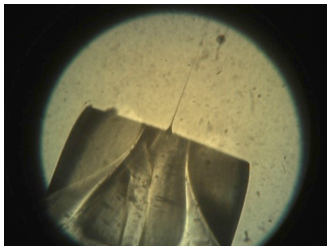

Figure 7: In vacuum jetting of a capillary with asymmetric bore inside a square ID glass tube. Since the cone tip of the liquid capillary is centered inside the gas aperture, the jet emerges straight. The liquid cone attached to the glass cone tip is visible. Square glass tube OD: 0.6mm (wall to wall). Gas exit aperture diameter ~100 micron. **[2]**

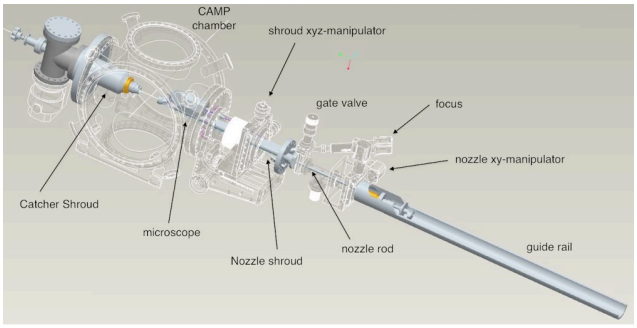

Figure 8: Differential pumping system for the liquid jet injector. It consists of two parts, the catcher shroud and the nozzle shroud, which can be decoupled and retracted via a bayonet coupling. This allows the use of fixed samples without breaking the vacuum.

5 CHALLENGES AND ADVANTAGES

The primary challenge which we face in this project is the requirement for each type of sample to undergo characterisation before it can be suitably trapped for x-ray crystallography. This is due to the fine balance between the optical trapping forces and the microfluidic flow rate. Depending on the geometry and composition of the nano crystals, this balance can vary substantially. The characterisation process while trivial, can be time-consuming. However, the optimum operational parameters once identified are re-usable and can be store in a database for later application.

Another challenge arises from the device being not designed for purpose. The optical tweezing device implemented for this project is an off the shelf unit, with components which were not designed for beamline applications. As such, the optofluidic chips require additionally modification, including x-ray aperture etching and replacing elements of the microfluidic channel with X

ray compatible alternatives. However, the implementation of this device is also one of the major advantages this project aims to highlight. Because of its elegant simplicity, and semiconductor manufacturing process, optofluidic chips are easily replaceable and open up the possibility of research specific customisation.

6 SYSTEM SETUP

The system setup follows closely the original optofluidic pattern, however two additional input reservoirs have been added. These are the Sample reservoir, the buffer reservoir and the reagent t reservoir. The buffer reservoir can contain either the sample buffer (saline buffer in the case of protein crystals) or deionised water. The principle is that it provides a reaction neutral flushing medium to expel superfluous particles from the chip which have not been trapped. This exact nature of this flushing medium is dependent on the sample being analysed also must not affect the reagent.

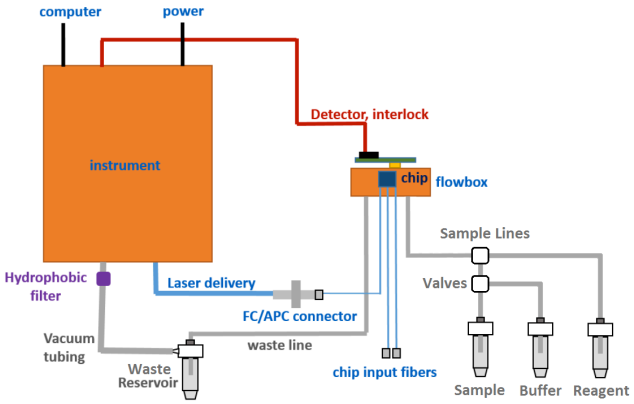

Figure 9. A schematic of the modified optofluidic system.

The process of operating an optically tweezed time-resolved diffraction experiment is demonstrated below in Figure 10.

First, the sample particles are introduced into the microfluidic channel and are trapped within the evanescent fields. The remaining particles are flushed out by a neutral medium. Next, the reagent is introduced, allowing it to react with the trapped particles. The remaining reagent is then flushed out once the reaction has started. Finally, the reacted particles are released and flushed out of the optofluidic chip.

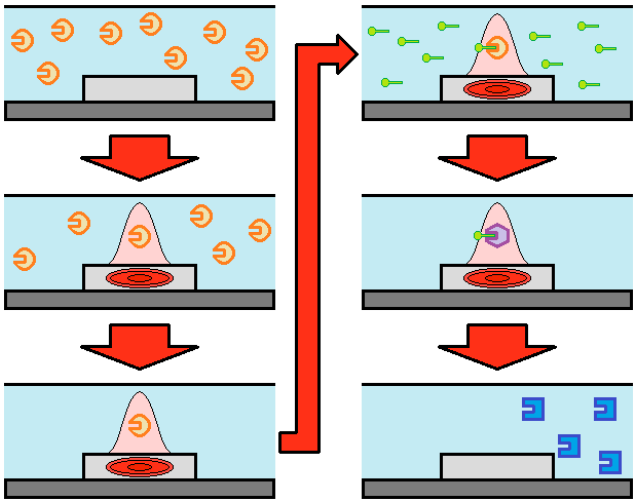

Figure 10. Diagram representing the steps in an a time resolved optical tweezing experiments.

7 CONCLUSIONS

This methodology for handling nanocrystals in its native environment for presentation to X-ray beams at both synchrotron and XFEL light sources is showing great promise. The addition of the ability to perform time-resolved experimentation, pushes this loading alternative to the forefront of x-ray crystallography technology. As the reaction takes place in a closed microfluidic system, very little sample is required and the diffraction can be undertaken at room temperature. The additional advantage, the sample is stationary, unlike other proposed beamline sample loading alternatives such as liquid jets. This reduces sample waste, and has the potential to increases measurement accuracy. This system particularly lends itself toward the study of Beta Lactamase and it reaction to Nitrocefin.

REFERENCES

[1] Neutze R, Opportunities and challenges for time-resolved studies of protein structural dynamics at X-ray free-electron lasers. Philos Trans R Soc Lond B Biol Sci. 2014 Jul 17;369(1647):20130318. doi: 10.1098/rstb.2013.0318.

[2] Spence, J. C. H., Weierstall, U. & Chapman, H. N Xray lasers for structural and dynamic biology. Reports on Progress in Physics 75, doi:10.1088/0034-4885/75/10/102601 (2012)