In cellulo serial crystallography of alcohol oxidase crystals.

A crystal factory inside yeast cells.

Developments in X-ray free-electron laser technologies and serial crystallography techniques have opened up new opportunities for studying challenging protein systems and complexes. Cell organelles known as peroxisomes naturally produce protein crystals in their interior. We show that it is possible to study crystals *in situ* in their native cell environment with advanced X-ray sources and see potential for harnessing this natural process for investigating proteins that are difficult to study with traditional methods.

Crystallography is an effective method for producing detailed atomic 3D models of proteins. But while crystallography is one of the most powerful methods in the structural biologist's toolbox, producing protein crystals in preparation for crystallographic experiments remains a bottleneck. There is no patent protocol for the crystallisation of proteins, and especially larger proteins, protein complexes and membrane proteins are notoriously difficult to crystallise – a process that can be extremely time and resource intensive. Large protein complexes will often produce minute crystals that are too fragile to be handled in the traditional way at the beamline. Radiation produced at X-ray free-electron lasers (XFELs) with brilliant X-ray pulses lasting only a few femtoseconds offers new opportunities for probing the 3D structures of protein species that as yet have been difficult to study at the synchrotron. For such samples, serial data collection strategies [1,2] have recently been developed to aid the data collection from many thousands of small crystals that are often easier to obtain than the large crystals needed for conventional crystallography experiments. These recent advances in XFEL technologies have sparked an interest in exploring other techniques producing large numbers of small crystals of biological macromolecules.

We explored the observation that protein crystals sometimes spontaneously form within cells and organelles [3]. Cell organelles known as peroxisomes are membrane bound organelles found in eukaryotic cells. Through our work with peroxisomes we knew they produce crystals in their interior, which are used to isolate and pack the toxic processes of fatty acid degradation. In the yeast *Hansenula polymorpha* (Hp), alcohol oxidase (AO) enzymes break down methanol into useful byproducts. These proteins are produced in such high numbers in peroxisomes that, to use the limited space most efficiently, they assemble into a tightly packed crystalline arrangement. We wondered whether this natural process could be harnessed to produce protein crystals of our own choice that could then be measured *in situ*, thereby circumventing the need for crystallisation trials and adding an additional tool

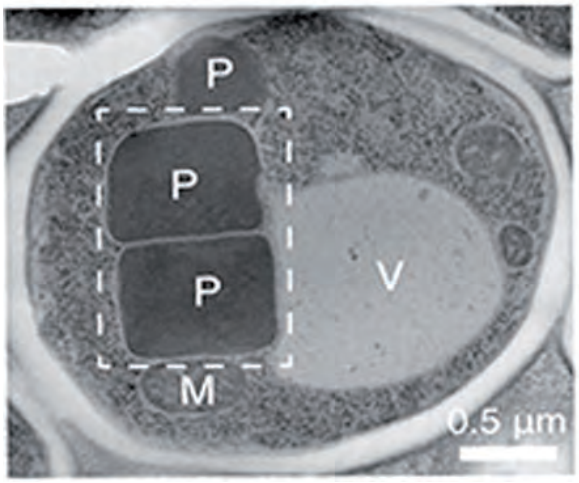

Figure 1

Electron micrograph of a wild type *Hansenula polymorpha* cell containing crystalline alcohol oxidase in electron-dense peroxisomes (P) seen next to mitochondria (M) and a vacuole (V).

to the structural biologist's toolbox. But first we needed to test whether this system can actually be used for crystallography experiments. Diffraction data of the Hp AO complex have only been observed to very low resolution to date. The small dimensions of the Hp peroxisomes make it an ideal candidate for testing the possibilities of XFELs for this purpose.

In order for this cellular process to be suitable for diffraction experiments, a uniform size and number of peroxisome crystals per cell is necessary, i.e. one large crystal per cell. Naturally occurring crystals are variable in size and number, however colleagues at the University of Groningen (NL) identified a mutant strain of Hp that only produces a single crystal filling out the entirety of the cell space. Yeast cultures were then grown on a methanol rich medium to induce the growth of AO crystals. Cell suspensions of the wild type and mutant Hp strains were tested at the EMBL operated macromolecular crystallography beamline P14 at PETRA III. Powder patterns

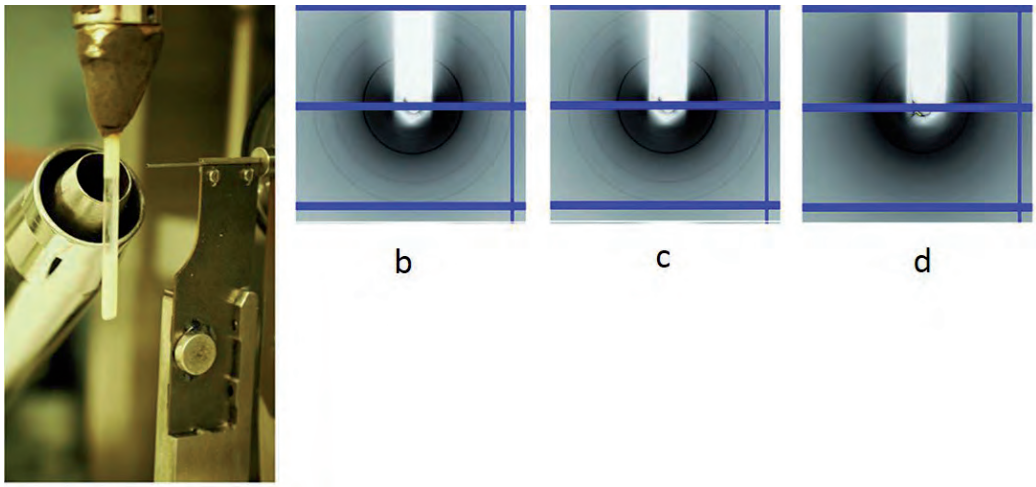

Figure 2

(a) Setup for powder diffraction experiments with cell and peroxisome suspensions on the P14 beamline at PETRA III showing from left to right the nozzle of the cooling device, the capillary with the sample and the pinhole of the X-ray beam. X-ray powder diffraction patterns are shown for (b) wild-type, (c) peroxisome and (d) cell suspensions.

were collected, visualised and analysed suggesting that the peroxisomes contained protein crystals.

In a next step at the Coherent X-ray Imaging (CXI) experiment at the Linac Coherent Light Source (LCLS), we tested the diffraction properties of the AO crystals *in situ* within intact cells, and within the peroxisomes which were extracted from the yeast cells. Being such a novel experiment, in collaboration with colleagues from the Chapman group at the Center for Free-Electron Laser Science (CFEL) and the University of Hamburg, we first had to optimise the conditions and methods for introducing the peroxisome and cell suspensions into the X-ray beam. We worked with a gas dynamic virtual nozzle (GDVN) to deliver the samples as a thin liquid jet into the X-ray beam. During initial test runs while using purified peroxisomes stored in sucrose buffers, we found that the sticky sucrose solutions rapidly clogged the nozzle resulting in instable jets. We therefore switched to using a sorbitol solution which worked well with the GDVN.

Diffraction patterns were obtained by exposing a fully hydrated stream of cells to X-ray pulses of 30 femtoseconds and energy of 7.925 keV. We collected data sets for suspensions both of intact cells and isolated peroxisomes. Although occasional diffraction patterns were found for the peroxisome suspensions, the very low resolution and the poor overall quality of the data meant we could not process these data sets any further. The data collected from the cell suspensions were more encouraging. While the majority of the diffraction patterns were restricted to 30 Å, resolutions of 6 Å were also seen.

We have been able to show that it is possible to obtain diffraction patterns from a large protein complex that has been crystallised in its native environment. HpAO is a challenging project for structure determination. Based on estimates from previous studies we believe the size of the crystals within the peroxisomes to be significantly smaller than grown in the traditional way in the laboratory. Hence we feel obtaining 6 Å resolution data from sub-µm sized crystals is a significant achievement for such a challenging sample using this novel method. Long term, we hope to be able to harness the natural ability of the peroxisome to also produce crystals from other proteins. Once developed, we see potential for the use of *in cellulo* serial crystallography as an additional complementary method for studying the atomic structure of difficult to crystallise proteins.

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