ESRF	**Experiment title:** Structural characterization of two fluorescent proteins supported by online microspectrophotometry	**Experiment number**: MX587
Beamline: ID14-eh2	**Date of experiment**: from 06/11/06 to 07/11/06 (from 04/12/06 to 05/12/06)	**Date of report**: 04/02/07
Shifts: 3 (+3)	**Local contact(s)**: John McGeehan	*Received at ESRF:*

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Report:

Experimental setup

Our primary aim was to allow the monitoring of fluorescence signal on a crystal placed on a beamline goniometer. This was made possible on the online microspec by the addition of the following items borrowed from the ESRF Cryobench laboratory: a bifurcated optical fibre, a 440nm laser diode, a pulse generator and an oscilloscope. We managed to obtain spectra with excellent signal-to-noise ratio, except for the left part of the spectra, because a lowpass filter (cutoff at 450nm) was required to remove the contribution of the excitation light. This setup allowed us to visualize the rapid bleaching (within seconds) of fluorescent protein crystals in the X-ray beam. The question whether this bleaching process is totally, or only partially, reversible is still open, since we observed recovery of the signal with a time constant of probably several tens of minutes.

Results for ECFP protein

We wanted to visualize the structural changes associated with blue light excitation of ECFP that we had previously observed on a microspec experiment at ID14-eh4. We did obtain similar results, yet not as clear. The observed changes include movements and disordering of aminoacid residue sidechains and water molecules in the close vicinity of the chromophore, but with no better resolution or occupancy. The probable reason for that is that, this time, we needed bigger crystals for getting sufficient resolution (ID14-eh2 has a beam less intense than ID14-eh4, and the maximal intensity in the ESRF ring was only 90 mA). The problem is that an ECFP crystal highly absorbs light at 440nm, which results in the fact that the core of crystal sees much less photons that the outer shell, in the case where the dimensions of the crystal are larger than 50 microns. There is still room for improvement (choice of thinner but wider crystal, centering of the X-ray beam on the incident light cone location, in a similar manner as for EosFP, see thereafter). At the same time, we would like to investigate into more details the effects of X-rays on ECFP fluorescence, which requires the use of the microspec in fluorescence mode.

Results for EosFP protein

Our aim was to study in detail the processes of photoconversion and photobleaching reactions of the fluorescent protein EosFP.We knew from our previous experiments performed at the Cryobench laboratory that no green-to-red photoconversion occurs when EosFP is illuminated at low temperature. One of the most interesting experiments has consisted in illuminating a crystal of EosFP by 355 nm laser light at 100K and to collect an X-ray dataset during the laser illumination (Fig. 1). In the present experiment, we aimed at exciting the molecules without photoconverting them and thus, obtaining the structure of an intermediate state in the photoconversion reaction.

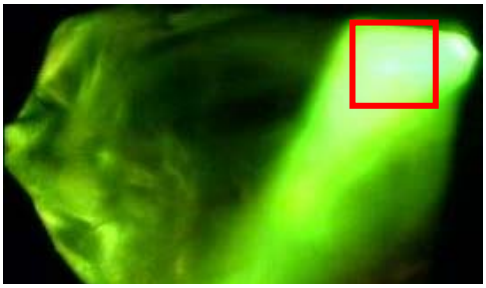

Figure 1 - Crystal of EosFP under illumination of a 355 nm laser source (fluorescent cone). A data collection is taken before the illumination and during this illumination (red square)

Both the ground state (i.e. non-illuminated crystal) and an excited state (i.e. illuminated crystal) datasets were of good quality (1.4 Å and 1.6 Å respectively) and allowed to calculate very precise difference electron density Fourier ($F_{obs}(2)$-$F_{obs}(1)$) maps at 1.6 Å.
An averaging of those maps taking into acount the 4-fold non-crystallographic symmetries of the EosFP tetramer was performed to increase the signal-to-noise ratio. These maps show very interesting results: the strongest peaks (negative) in the maps are located on three residues close to the chromophore. This suggests that these three residues move during the illumination in a concerted way, probably generating the displacement the whole chromophore (as seen with the negative and positive peaks on the chromophore).

These results shared similarities with the ones results obtained in our team on the protein ECFP. We can thus postulate that those movements are related to a photobleaching phenomenon, rather than the formation of an excited state in the photoconversion pathway. This is in accordance with our previous observation that EosFP can suffer from a slight photobleaching when strongly irradiated at 355 nm at 100K.

Conclusions

We have learned a wealth of information from this experiment, on both the technical and scientific sides. We showed that we could monitor online the fluorescence of a protein crystal with modification of the online microspec equipment. This allowed us to witness that X-rays had a dramatic effect on the signal. We would like now to investigate these effects in more details. We have confirmed the structural features of the ECFP photobleaching mechanism, yet not at better resolution nor better occupancy. Finally, we have observed for the first time structural changes associated with the excitation of EosFP fluorescence.

We are definitely willing to go on with such experiments, which represents a unique opportunity to apprehend the mechanism of fluorescents proteins. This constitutes a challenge in nowadays macromolecular crystallography at synchrotrons.

Note: The beam was lost shortly after midnight on November, 7th, because of problems that could not be addressed by us (problem with mirror). For that reason, our experiment had been rescheduled on December 4th by the MX BLOMs, which we warmly thank.