ESRF	**Experiment title:** Time-resolved small-angle scattering study of the R to T transion in hemoglobin	**Experiment number:** SC-1855
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Report:

Aim of this proposal was to develop time resolved Small and Wide Angle X-ray Scattering (SWAXS) and use this technique to study allostery in protein.
In particular we focused our attention on Hemoglobin (Hb). Hb is a tetrameric protein complex often used as model protein to study allostery. The interaction between subunits gives rise to cooperative ligand binding which is essential for the biological function of Hb since it enables substantial downloading of O_2 to the tissues in response to a relatively small partial pressure decrease.

We used Hb from human blood. It's well known that when one ligand molecule is photodissociated there are $\sim$ 50 % probability to recombine from the heme pocket (geminate rebinding) that occur in $\sim$ 50 ns. Moreover it's known that the dissociation of 2 or more ligands is necessary for the Hb to undergo the quaternary transition. This means that the geminate recombination will lower the fraction of molecules undergoing the transition we are interested in. In order to make this probability higher we used a 150ns long laser pulse to excite the protein. In this way we have multiple changes to expel the ligand from the protein, giving higher yield of relaxing proteins. The laser used is the "Evolution X" that is placed inside the amplifier "Hurricane". The time jitter has been measured with a 6 GHz Le Croy oscilloscope and was $<$ 10ns. The negative point of this pump scheme is that it doesn't allow for time delays shorter than the luser pulse.

We think that this problem can be solved in the future, one the technique will be fully developed, by using Hb mutants that don't recombine geminately.
The laser spot used was 450 μm (along the x-ray path) and 150 μm perpedicular to it. The energy per pulse was 0.8 mJ.
The concetration used was 2 mM and was a compromise between signal from the protein and laser penetration/interference between proteins. The x-ray setup is depicted in figure 1. We use a cone (200 mm long) filled with He to reduce air scattering. The sample detector distance was 300 mm. Being x-ray photon starved we used a polychromatic incoming beam (FWHM 3%, centered at 15.5 keV). With this energy the accessible q range was 0.03 $\AA^{-1}$ (beamstop limited) - 1.7 $\AA^{-1}$ (ccd limited). We used 480 x-ray pulses per image at 5 Hz. To spread the laser heting problem we used a moving capillary. The data collection strategy was as follows: we alternate one image with laser excitation and one without and the we repeated the sequence 10 times. Due to the time spent in setting up and tuning the different parameters we had not time to collect an entire time series. So we focus our attention on a time delay of 30 μs where, according to the literature, there should be the deoxy-like quaternary structure.
We compared the measured signal (laser on − laser off) with a calculation based on the crystallographic structures R2 (ligated protein) and T (unligated one). The calculation have been done with Crysol. The results are shown in the figure. The comparison is very compelling and indicates they we are able to understand the structural informations.
We believe that this preliminary attemp can be considered very successfull. There are still a few open questions like: (1) effect of the laser heating on the difference map, (2) is the difference between the calculated map and the measured one due to the fact that we are using the crystallographic data ?, (3) Are there intermediated during the transition ? We will need more beamtime to address these questions but we think that we are on the right track to develop a new and powerfull technique that will open a new field in the biophysical related research.