Measuring Solvent Content of Biological Crystals Using Fluorescence Recovery after Photobleaching

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Abstract: We work out a novel protocol for measuring the solvent content (the fraction of crystal volume occupied by solvent) in biological crystals by the technique of fluorescence recovery after photobleaching (FRAP). Crystals of proteins with widely varying known solvent content (lysozyme, thaumatin, catalase, and ferritin) were grown in their native solution doped with sodium fluorescein dye and hydroxylamine (to prevent dye from binding to amine groups of the proteins.) The crystals were irradiated by a broadband, high intensity light through knife slits, leaving a rectangular area of bleached dye within the crystals. By fitting the fluorescence recovery to 3D computational models obtained from the diffusion equation, we were able to obtain the diffusion coefficient within these crystals. Measuring the flow of dye out of the bleached area allowed us to construct a curve relating the diffusion coefficient of dye to the channel size within the crystals. This curve may be used to measure the solvent content of any biological crystal in its native solution and help determine the number of proteins in the crystallographic asymmetric unit cell in x-ray structure solving procedures.

Motivation: Understanding inter-diffusion within the pores of macromolecular crystals has vast applications: for exploiting hindered reaction rates within the crystal for chemical catalysis; for understanding soaking protocols prior to x-ray diffraction studies; and for understanding basic physics of diffusion in confined spaces. In this study, we measure the diffusion of fluorescent dye within crystals using the microscopy technique of FRAP and use the results to deduce the solvent content of the crystals.

Methods: We grow (micron size) crystals of 4 proteins on a microscope slide using standard methods. We dope the native solution with flourescein sodium dye and hydroxylamine, and briefly irradiate the crystal through a set of knife slits by high intensity light, leaving a strip of photobleached dye within the crystal.

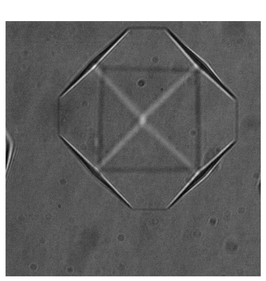

Figure 1: Microscope image of a Lysozyme Crystal in transmission mode.

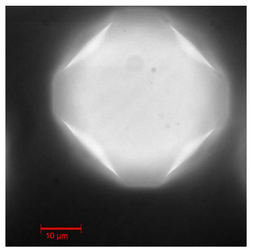

Figure 2: Lysozyme crystal viewed in fluorescence mode.

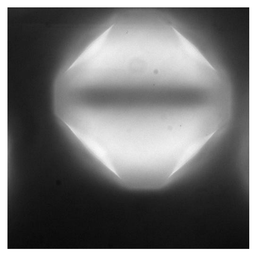

Figure 3: Lysozyme Crystal after Photobleaching

Diffusion:
We expect that diffusion of dye within the crystal relates to the crystal pore size through which it can flow. We consider the solution to the diffusion equation:

$$\frac{\partial C}{\partial t} = D(\nabla^2 C)$$

where D is the diffusion coefficient of the bleached dye out of the rectangular spot. Using rectangular boundary conditions (the dye concentration is held fixed on the perimeter), we solve the equation analytically using the Fourier method. We expect that D will depend on the pore size (and thus the solvent content) of the crystal.

$$C(x,y,z,t) = \sum_{n,m,r} A_{nmr} \sin\left(\frac{n\pi x}{L}\right) \sin\left(\frac{m\pi y}{M}\right) \sin\left(\frac{r\pi z}{P}\right) \times e^{-\pi^2\left(\left(\frac{n}{L}\right)^2+\left(\frac{m}{M}\right)^2+\left(\frac{r}{P}\right)^2\right)Dt} \quad \text{eq. 1}$$

The coefficients A_{nmr} were obtained by Fourier analyzing the freshly bleached image (t=0s).

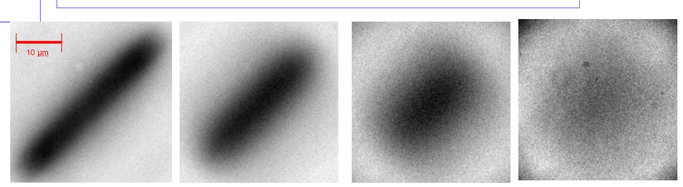

Figure 4: Lysozyme Crystal undergoing fluorescence recovery: at time 0, 40, 100and 400 s

$$A_{nmr} = \frac{8}{LMP}\iiint_0^{L,M,P} \psi(x,y,z,0) \sin\left(\frac{n\pi x}{L}\right) \sin\left(\frac{m\pi y}{M}\right) \sin\left(\frac{r\pi z}{P}\right) dxdydz \quad \text{Eq. 2}$$

We then evolve the concentration profile with time and fit it to the observed intensity profile in later images as the fluorescence recovers, using D as the parameter.

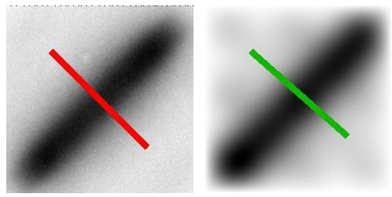

Figure 5: Comparison of observed initial image of the recovering area (Left), and the Fourier constructed surface (right). The red and green lines are the line profiles used for fitting.

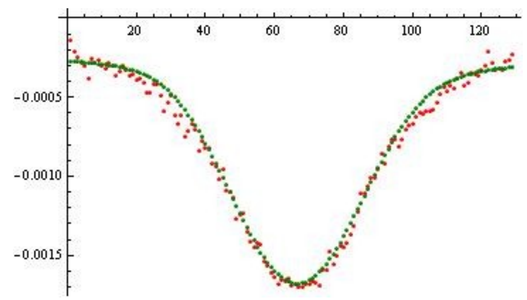

Figure 6: Intensity plot along the profile lines in Fig 5.

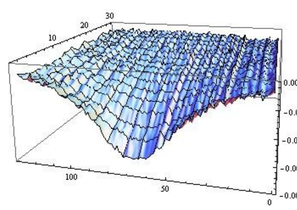

Figure 7: Stack of line profiles from recovering crystal. Depth

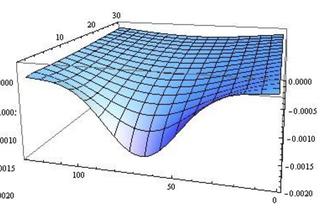

Figure 8: Stack of line profiles of from Fourier reconstructed

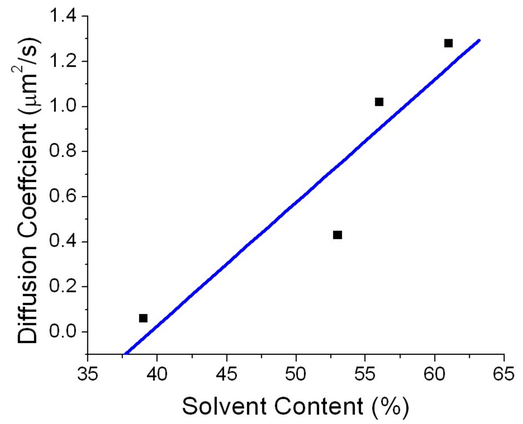

Figure 9: Plot of Solvent Content vs. Measured Diffusion Coefficients. The blue line is a guide to the eye.

Results: Table 1 lists the measured diffusion coefficient of the fluorescent dye in each of the crystals and the results are plotted in Figure 9. Consistent with other studies of small molecule diffusion within biological crystals, we find the fluorescein diffusion ranges from 0.06 to 1.28 $\mu m^2/s$ within the four crystals of different porosities; these values are several orders of magnitude lower than the diffusion of 490 $\mu m^2/s$ of the dye in bulk water.

Conclusion: The diffusion coefficient of fluorescein within crystals of lysozyme, catalase, thaumatin, and ferritin depends on the channel size of these crystals and varies by an order of magnitude. The extreme sensitivity of the diffusion coefficient to the size of the pores makes our FRAP method viable for measuring the solvent content of any biological crystal in its native environment. Determining the solvent content is a precursor step during x-ray structure solution procedures and out method measures solvent content in the native environments. The varying transport properties of dye within different crystals may also be useful for practical applications that require fine-tuning of reaction kinetics based on diffusion speeds, e.g., in application requiring precision parameters for reaction catalysis.

Protein	PDB Code*	Solvent Content (%)†	Flourescein intra - crystal Diffusion ($\mu m^2/s$)
Lysozyme	1lz8	39	0.06
Catalase	8cat	53	0.43
Thaumatin	1ly0	56	1.02
Ferritin	1ier	61	1.28

Table 1: List of protein crystals, their calculated solvent percent by volume, and the measured diffusion coefficients
*The unit-cell parameter and symmetry of our crystals were identical to these depositions
† Calculated following the formalism of Matthews (1968) [1]

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Refrences:
[1] Kmetko, J., Husseini, N. S., Naides, M., Kalinin, Y. & Thorne, R. E. (2006). Acta Cryst. D62, 1030–1038.

[2] Soumpasis, D. M. 1983. Theoretical analysis of fluorescence photobleaching recovery experiments. Biophys. J. 41:95–97.

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