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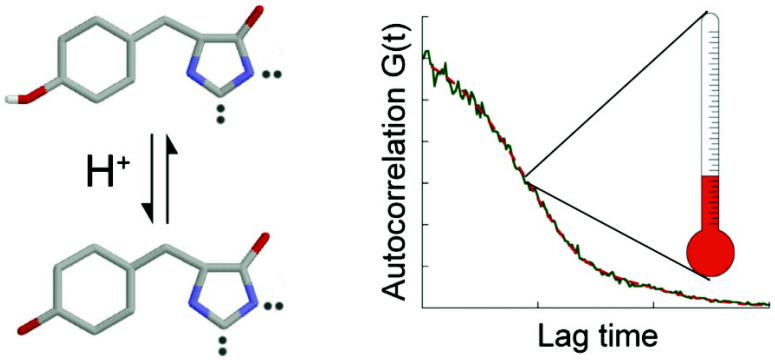
J. Am. Chem. Soc., **2007**, 129 (34), 10302-10303• DOI: 10.1021/ja0715905 • Publication Date (Web): 08 August 2007



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Published on Web 08/08/2007

A Molecular Thermometer Based on Fluorescent Protein Blinking

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With the current trend toward a miniaturization of chemical systems comes the need for precise physical measurements that will lead to an exact depiction of microfluidic environments. Traditionally, spatially resolved temperature measurements in fluids have been achieved using diffraction-limited optics to image dyes such as rhodamine B whose fluorescence quantum yield depends on temperature.[1] This method has been applied to observe temperature gradients in microfluidic channels[2,3] and in biological systems.[4] However it does not permit quantitative measurements because local dye concentration and background fluorescence also affect fluorescence intensity. To resolve this issue, methods based on the comparison between emission at different wavelengths[5] or on fluorescence lifetime measurements[6] have been proposed. Here, we propose another approach relying on the detection of the blinking of fluorescent proteins using fluorescence correlation spectroscopy (FCS). To demonstrate the usefulness of this method, we have applied it to the measurement of laser induced heating in thin liquid samples.

Many fluorescent proteins have the capacity to alternate between states with distinct fluorescence properties. In the case of the enhanced green fluorescent protein (EGFP), protonation of the Tyrosine-66 hydroxyl group on the chromophore brings the molecule from a deprotonated state, which is fluorescent upon 488 nm excitation, to a protonated state, which is mostly nonfluorescent under the same conditions. This characteristic has been exploited to design variants of the protein that can be used as genetically encoded molecular pH indicators.[7] The fluctuations in the fluorescence signal due to this reversible protonation reaction are referred to as blinking. The associated submillisecond relaxation time, which can be measured using FCS, is as expected very pH-sensitive.[8] We show here that this blinking also strongly depends on temperature, a property that makes GFP derivatives attractive candidates for use as molecular thermometers.

The temperature dependence of the relaxation time associated with the blinking of the EGFP is shown in Figure 1. To measure this dependence, protein samples were prepared by diluting purified recombinant EGFP (BioVision) to nanomolar concentrations in CP buffer (10 mM citric acid, 100 mM potassium phosphate) at different pH. The solution was then placed in a small chamber (thickness $\approx 100\ \mu$m, volume $\approx 10\ \mu$L) made of two microscope cover slips spaced by Parafilm and sealed with wax. Blinking relaxation times were obtained using a home-built FCS instrument already described elsewhere.[9] EGFP fluorescence was excited with the 488 nm line of an argon laser, keeping the excitation power at the sample below 80 μW. The temperature of the liquid was adjusted by controlling the temperature of both the stage and the water objective used for these experiments with two separate Peltier elements (Linkam Scientific Instruments). The resulting autocorrelation functions (each corresponding to a 60−180 s measurement) showed two separate decays, one due to the diffusion of the proteins

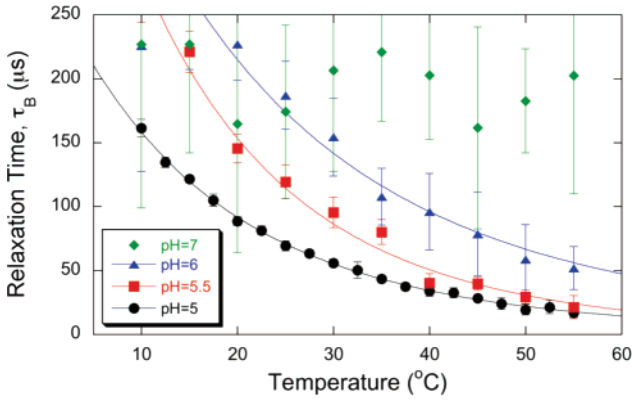

Figure 1. Calibration curves showing the temperature dependence of the relaxation time associated with EGFP blinking for different pH values. Data sets obtained for pH ≤ 6 have been fitted using eq 1.

in the observation volume (~0.5 ms) and the other due to their blinking (~0.1 ms). They were analyzed for lag times above 10 μs using a model taking into account both processes. Two parameters characterizing the blinking process are extracted from this analysis: the relaxation time associated with protonation, τ_B, and a coefficient, B, which in the absence of noise represents the average fraction of fluorophores found in the nonfluorescent protonated state.[8]

Simple protonation and deprotonation reactions should follow Arrhenius' law, in which case we expect at low pH (pH ≤ 6)

$$1/\tau_B = 10^{-pH}A_p \exp(-E_p/RT) + A_d \exp(-E_d/RT) \quad (1)$$

$$B/(1-B) = 10^{-pH}(A_p/A_d) \exp(-(E_p - E_d)/RT) \quad (2)$$

Using these relations to fit our data, we found that the values of the activation energies, E_p and E_d, and the frequency factors, A_p and A_d were constant within error at very low pH (pH ≤ 5.5) as expected for an external protonation reaction. These values (E_p = 9.2 ± 0.3 kcal/mol and E_d = 10.3 ± 0.5 kcal/mol for pH = 5) are smaller than those measured using pH jump experiments,[10] but consistent with previous FCS measurements.[8]

Our measurements show that the relaxation time τ_B is very sensitive to temperature changes at low pH. In contrast, the values obtained for τ_B are not affected by fluorophore concentration, motion, or photobleaching, by the level of background fluorescence, by the excitation power, or by the size and shape of the detection volume. The relaxation time is therefore a useful parameter to exploit for temperature measurements. It does however depend on buffer composition (including viscosity) and pH,[8] so a new calibration curve must be obtained for each new buffer condition. The precision obtained on the value of τ_B increases at low pH because the nonfluorescent protonated form of the protein becomes

10.1021/ja0715905 CCC: $37.00

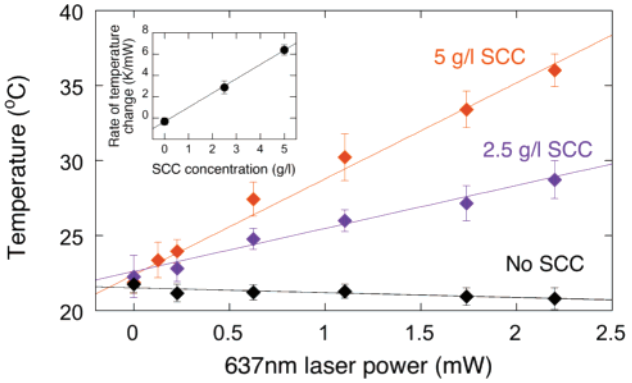

Figure 2. Increase in temperature at the focus of a 637 nm laser beam for solutions containing different concentrations of SCC. Lines represent linear fit of the data.

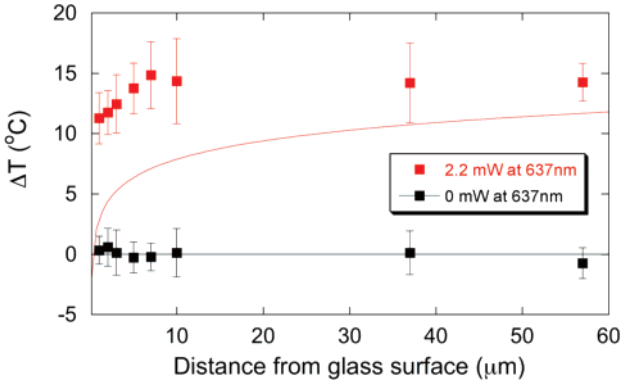

Figure 3. Temperature change at the laser focus in a 5 g/l SCC solution as a function of the distance between the laser focus and the glass cover slip, in the presence and in the absence of the 637 nm laser radiation. Solid lines are theoretical predictions from ref 11 (ϵ = 35 cm^{-1}, K = 5.86 W·K^{-1}·cm^{-1}).

predominant, which increases the relative amplitude of the blinking term in the autocorrelation function.

As a first application of this molecular thermometer, we characterized the heating caused by a focused laser beam passing through a thin absorbing liquid sample, a situation often encountered in single cell or single molecule manipulation by optical tweezers. For this experiment, we used a commercial FCS instrument (Insight Cell, Evotec Technologies), which allowed combining two laser beams through the same optical fiber. We used the 637 nm radiation of a continuous-wave laser diode to produce a temperature increase in solutions of sodium copper chlorophyllin (SCC), a derivative of chlorophyll with a high extinction coefficient in the near-infrared. A second beam, the 488 nm line of an Ar laser, was used to excite EGFP fluorescence. The power of that second laser was kept very low, $P \approx 80$ μW at the sample. The studied samples were prepared by dissolving SCC (Sigma) in CP buffer (pH = 5) supplemented with 100 g/L BSA and EGFP. The solutions were placed in ~100 μm thick chambers as described above. A calibration curve was acquired for each different SCC concentration. The temperature at the focus of the 637 nm laser line was inferred from the measured relaxation time of EGFP at that same position (10−20 s measurements).

The measured temperature increase is shown in Figure 2 in the case where the 637 nm laser was focused far from the glass cover slip. We expect a temperature change $\Delta T \approx \epsilon P/K$, where P is the incident laser power and ϵ and K are the extinction coefficient and thermal conductivity of the solution, respectively. Indeed, our results show that the increase in temperature at the laser focus is directly proportional to the power of the 637 nm radiation, and that it is also directly proportional to SCC concentration, with no detectable increase in the absence of SCC. In addition, our measurements show that the increase in temperature due to laser heating is reduced as the laser focus is brought closer to the glass surface of the sample chamber (Figure 3). This is because the light absorption in the glass is negligible compared to that in the liquid, and because the cover slip, which is in direct contact with the water objective, acts as a heat sink. Far from the glass surface, a model taking into account light absorption and heat dissipation accurately predicts the temperature increase we measured.[11] However this simple model fails close to the surface, due to the difficulty of accurately taking boundary conditions into account. Our method on the other hand allows an accurate temperature measurement at the cover slip. The blinking properties of EGFP are not modified in the proximity of the glass surface, as proved by the fact that in the absence of laser heating the same temperature is correctly recovered in the bulk and at the surface of the sample (Figure 3). This has interesting implications for the exact monitoring of temperature at the surface of microfluidic channels containing tethered active biomolecules.

Using fluorescent protein blinking as detected by FCS to measure temperature presents multiple advantages. It provides absolute temperature measurements, independent of experimental conditions when using typical FCS conditions (excitation intensity below 200 μW, fluorophore concentration below 100 nM). It can be used to characterize steep temperature gradients, such as those expected in microfluidic and microcapillary flows. It can be used at a liquid/solid interface as long as the blinking properties of the proteins are not modified by presence of the solid surface. It is noninvasive providing an air objective is used instead of a water objective. The blinking properties of EGFP, however, remain sensitive to pH and buffer composition,[8] and therefore one limitation of the method is that a specific calibration curve should be obtained for each buffer condition. Finally, we note that this method has the potential to be applied directly in biological samples using genetically encoded fluorescent protein fusions. Using appropriate variants of the protein with an observable temperature dependence at pH = 7.4 should lead to precise temperature determination under normal physiological condition.

Acknowledgment. This work was supported by the Canadian Institute for Photonic Innovations (CIPI) and by the Natural Sciences and Engineering Research Council of Canada (NSERC) in particular through its Canada Research Chair program.

Supporting Information Available: Details on data fitting, thermodynamic parameters obtained at different pH, and reproducibility of the calibration curves. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Karstens, T.; Kobs, K. *J. Phys. Chem.* **1980**, *84*, 1871.
(2) Erickson, D.; Sinton, D.; Li, D. *Lab. Chip* **2003**, *3*, 141.
(3) Ross, D.; Gaitan, M.; Locascio, L. E. *Anal. Chem.* **2001**, *73*, 4117.
(4) Kato, H.; Nishizaka, T.; Iga, T.; Kinosita, K., Jr.; Ishiwata, S. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9602.
(5) Sakakibara, J.; Adrian, R. J. *Exp. Fluids* **1999**, *26*, 7.
(6) Jeon, S.; Turner, J.; Granick, S. *J. Am. Chem. Soc.* **2003**, *125*, 9908.
(7) Miesenbock, G.; De Angelis, D. A.; Rothman, J. E. *Nature* **1998**, *394*, 192.
(8) Haupts, U.; Maiti, S.; Schwille, P.; Webb, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13573.
(9) Banks, D. S.; Fradin, C. *Biophys. J.* **2005**, *89*, 2960.
(10) Saxena, A. M.; Udgaonkar, J. B.; Krishnamoorthy, G. *Protein Sci.* **2005**, *14*, 1787.
(11) Peterman, E. J. G.; Gittes, F.; Schmidt, C. F. *Biophys. J.* **2003**, *84*, 1308.

JA0715905