

## Influence of Viscosity and Molecular Size on Temperature Sensitivity of Fluorescence Anisotropy

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The temperature measurement of liquids is essential in many fields such as the thermal management of a chemical process or material synthesis. It is relatively easy to measure the liquid temperature on the macroscale, but temperature imaging on the micro/nanoscale is still challenging. Conventional methods such as the use of thermocouples and resistance temperature detectors and laser-induced fluorescence have drawbacks when applied to microfluidic temperature imaging techniques. In the present work, fluorescence anisotropy (FA) was used as a liquid temperature measurement method on the microscale. FA has an advantage over conventional methods of intrinsic normalization of the light intensity, which enables ratiometric measurement even when using a single wavelength from a fluorophore. We measured FA values in liquids of different viscosities and temperatures using a spectrofluorometer having two rotational polarizers, then obtained the temperature sensitivity of FA. The temperature sensitivity of FA was also theoretically investigated using the derivative of Perrin's equation, which relates FA, viscosity, temperature, fluorescence lifetime, and molecular size. The experimental results show that each molecule has an optimal viscosity range indicating the maximum temperature sensitivity and that fluorescein isothiocyanate–dextran conjugates with smaller molecular weights have higher sensitivity. Also, reasonable agreement between experimental and theoretical results was confirmed. Consequently, it was clarified that the temperature sensitivity of FA can be controlled by labeling to adjust the required viscosity range of the sample solution and that theoretical estimation provides qualitative guidance for FA-based thermometry.

### 1. Introduction

Microfluidic temperature measurement is in high demand. One example is the temperature imaging of a liquid thermal interface material (TIM) inserted between two solid surfaces to reduce the thermal contact resistance between them.<sup>(1)</sup> Since it is challenging to obtain the

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temperature information of a liquid sandwiched by two solids,<sup>(2)</sup> the interfacial temperature is often estimated from ambient temperature information. However, this estimation method includes large uncertainties, especially when using highly thermally conductive TIMs.<sup>(3)</sup> As shown in this example, there are technical limitations in estimating temperature from the outside. Other examples can be seen in various biomedical microdevices or lab-on-a-chip applications based on microfluidic technology.<sup>(4)</sup> Temperature measurement of microscale liquids plays an important role in material synthesis, reaction control, biosensing applications,<sup>(5)</sup> and advanced health monitoring.<sup>(6)</sup> The fabrication of a micro-thermocouple, which utilizes the Seebeck effect to measure the temperature in a device, is only sometimes helpful because of the invasive nature of the technique.<sup>(7)</sup> Laser-induced fluorescence (LIF) is a technique that uses the temperature dependence of fluorescence intensity, namely, the thermal quenching of fluorophores, to measure the liquid temperature noninvasively.<sup>(8,9)</sup> LIF can be used only with a uniform liquid thickness and illumination intensity because it is difficult to distinguish the effect of nonuniformities from the signal. Although two-color LIF can overcome the drawback of such nonuniformities by adding temperature-insensitive fluorescent molecules in the liquid,<sup>(10)</sup> the concentration ratio should be the same in the whole field.

Our group has focused on the fluorescence anisotropy (FA) of fluorophores as an indicator of liquid temperature.<sup>(11–13)</sup> This technique analyzes the polarization characteristics of the fluorescence emission of a probe molecule. FA is independent of the solution pH<sup>(14)</sup> and is not affected by a nonuniform illumination intensity, molecular concentration,<sup>(15)</sup> or liquid film thickness, which may cause significant errors in LIF measurement. We previously reported on the variation of FA with temperature. In our previous study, we clarified that the size of the thermal probe influences the temperature coefficient.<sup>(11)</sup> In other studies, the temperature dependence of FA for various molecules was investigated on the basis of Perrin's law.<sup>(16,17)</sup> Recently, the important effect of the temperature-dependent viscosity on the rotational correlation time has been reported.<sup>(18)</sup> However, a detailed investigation of the temperature sensitivity of FA, similarly to that of the temperature coefficient, has not yet been performed. To develop FA-based temperature measurement as a promising method for liquid thermometry, we consider that guidelines for the selection and development of probe molecules are necessary. In the present study, the effects of the solution viscosity and the size of fluorescent molecules on the temperature sensitivity of FA were investigated. The temperature sensitivity of fluorescein and its derivatives with different Stokes radii (related to the hydrodynamic volume) and various solution viscosities was examined on the basis of Perrin's law.

## 2. Theory

### 2.1 Fluorescence anisotropy and temperature sensitivity

When a fluorescent molecule is irradiated with linearly polarized light, the distribution of excited fluorescent molecules becomes aligned along the polarization direction of the excitation light as shown in Fig. 1.<sup>(19)</sup> The fluorescence intensity has anisotropy according to the directional distribution of the excited molecules. Consequently, the relaxation of FA is strongly influenced

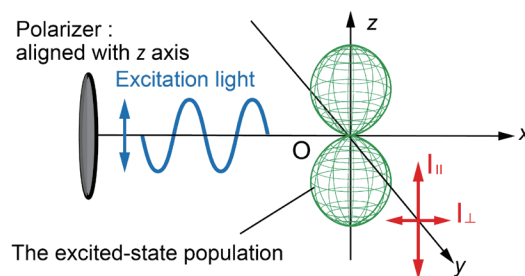


Fig. 1. (Color online) Schematic of population of excited fluorescent molecules with excitation light along z axis.

by the distribution of absorption and emission moments, the rotational Brownian motion of the molecules, and the transfer of excitation energy between fluorescent molecules.<sup>(12)</sup> Since rotational Brownian motion is temperature-dependent among the parameters that affect FA, it is possible to extract the temperature information of the molecules in the liquid from FA. FA,  $r$ , is defined as the degree of polarization of the fluorescence emission divided by the total fluorescence intensity as follows.

$$r \equiv \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (1)$$

Here, the fluorescence component parallel to the excitation light is  $I_{\parallel}$  and the perpendicular component is  $I_{\perp}$ .  $G$  is the apparatus constant for the polarization dependence of the transmittance.<sup>(16)</sup>

FA is normalized by the total fluorescence emission intensity; therefore, this parameter can be used to measure the liquid temperature while canceling the effects of the nonuniform excitation light intensity, molecular concentration, and liquid thickness, which would cause uncertainty in the conventional LIF and two-color LIF techniques. In this study,  $r$  was obtained for different solution viscosities and temperatures, then the temperature sensitivity was determined.

## 2.2 Perrin's equation and temperature sensitivity

To compare the temperature sensitivity of FA with the conventional theory, we used Perrin's equation, defined by

$$r \equiv \frac{r_0}{1 + \frac{\tau k_B T}{V\eta}} \quad (2)$$

Perrin's equation is based on the time average of the random rotational diffusion of spherical molecules<sup>(16)</sup> and is a well-known expression relating FA  $r$ , temperature  $T$ , viscosity  $\eta$ , and so forth. Here, FA in the absence of molecular motion is  $r_0$  (it is also called the fundamental

anisotropy). The fluorescence lifetime is  $\tau$ , Boltzmann's constant is  $k_B$ , and the hydrodynamic volume of the fluorescent molecule is  $V$ .

Equation (2) explicitly indicates that FA is a temperature-sensitive parameter.<sup>(11,18)</sup> It can also be confirmed that FA depends on the sample fluorescence lifetime, molecular size, and viscosity. In this study, we compared the temperature sensitivity of FA by changing the molecular size as a probe-dependent parameter and the viscosity as a solvent-dependent parameter. Equation (3), obtained by the total differentiation of Eq. (2) with respect to  $T$ , was used to determine the theoretical temperature sensitivity of FA.

$$\frac{dr}{dT} = \frac{\partial r}{\partial T} + \frac{\partial r}{\partial \eta} \frac{\partial \eta}{\partial T} + \frac{\partial r}{\partial \tau} \frac{\partial \tau}{\partial T} = -\frac{r_0}{(1 + \tau k_B T / V \eta)^2} \frac{\tau k_B}{V \eta} \left( 1 - \frac{T}{\eta} \frac{\partial \eta}{\partial T} + \frac{T}{\tau} \frac{\partial \tau}{\partial T} \right) \quad (3)$$

### 3. Experimental Method

#### 3.1 Sample and medium

Glycerol solution was used as a solvent in this study, and the viscosity of the solution was adjusted from 12.1 to 142 mPa·s by changing the concentration of glycerin (50, 75, and 90 mass%) and by varying the temperature in the range of 25–55 °C. Fluorescein derivatives were employed as a temperature-sensitive molecular probe in this study because fluorescein is a well-known molecule with FA.<sup>(11,19)</sup> Here, fluorescein sodium salt (uranine, purchased from Fujifilm Wako) and fluorescein isothiocyanate (FITC) labeled with a polysaccharide dextran of different molecular weights, i.e., FITC–dextran conjugates (MW 10000, 20000, 40000, and 150000, purchased from Sigma-Aldrich), were used to investigate the effect of the molecular size on the temperature sensitivity of FA. The sample concentration was 0.01 mM under the diluted condition.

#### 3.2 Equipment

In this study, we calculated and compared the temperature sensitivity of FA using two of the methods described in Sects. 2.1 and 2.2. A spectrofluorometer (FP-8300, JASCO) was used to obtain  $r$  using Eq. (1). The excitation wavelength was 490 nm, and fluorescence spectra were measured in the range of 400–700 nm. The maximum value of the spectrum was used as the fluorescence intensity. Two polarizers with rotational polarization planes were placed in the optical path to extract the two orthogonal fluorescent components. The fundamental anisotropy  $r_0$  was obtained as an extrapolated value of the temperature-dependent FA at absolute zero. The fluorescence lifetime  $\tau$  was measured at various temperatures using a time-correlated single-photon counting method (Quantaaurus-Tau C11367–31, Hamamatsu Photonics). A rheometer (Discovery HR-1, TA Instruments) with a cone plate was used to measure the viscosity  $\eta$  of the solutions. The solution temperature was precisely controlled using a Peltier element embedded in the sample stage of each setup.

## 4. Results and Discussion

### 4.1 Experimental results

The temperature sensitivity was calculated from FA at each temperature obtained by the spectrofluorometer. Figure 2 shows the relationship between the temperature and FA of fluorescein sodium in 90 mass% glycerol solution. The third-order polynomial approximation by the least-squares method is also shown in the figure. We defined the derivative of this approximate function with respect to temperature at each measurement point as the temperature sensitivity of the sample; seven data were obtained from Fig. 2. The same process was applied to other samples, and the temperature sensitivity of each sample was obtained. Figure 3 shows the temperature sensitivity of FA for various viscosities of each sample. It is clearly indicated that each sample has viscosity dependence. Fluorescein sodium has a peak at a relatively high viscosity, and FITC–dextran samples have peaks at relatively low viscosities, whereas the peak viscosities are almost the same. The temperature sensitivity of fluorescein sodium exceeds that of FITC–dextran above a viscosity of 15.4 mPa·s. Among the FITC–dextran samples with different molecular weights, the temperature sensitivity depends on the molecular weight. Below

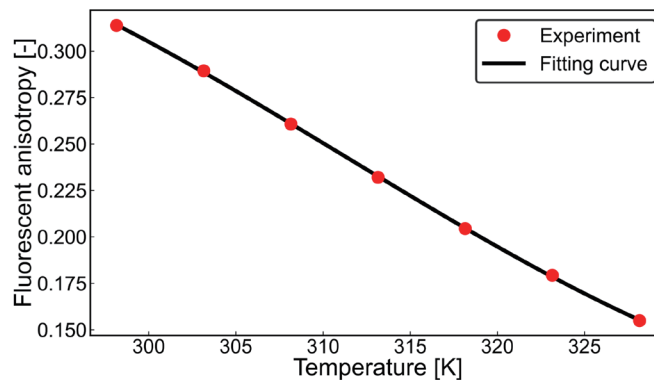


Fig. 2. (Color online) Temperature-sensitive FA of fluorescein sodium in 90 mass% glycerol solution.

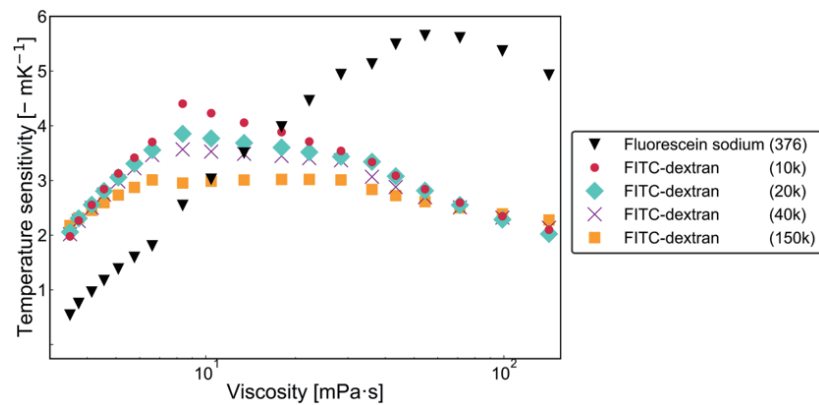


Fig. 3. (Color online) Measured temperature sensitivity of FA for fluorescein sodium and FITC–dextran.

a viscosity of 15.4 mPa·s, fluorescein sodium shows the lowest sensitivity, and the highest sensitivity was confirmed for FITC–dextran with a molecular weight of 10000. Below a viscosity of 5 mPa·s, there was no molecular weight dependence of the sensitivity, and the temperature sensitivity of FITC–dextran was approximately twice that of fluorescein sodium. Therefore, we can conclude that dextran labeling on fluorescein is effective in low-viscosity solutions, and smaller labeled molecules may improve the sensitivity around viscosities of 10 mPa·s. FA reflects the rotational motion of the molecules in the medium during the relaxation time of fluorescence emission.<sup>(19)</sup> When an excited fluorophore returns to ground state while emitting fluorescence, the temperature sensitivity decreases. On the other hand, the fluctuation of the fluorescence intensity with time leads to photoblinking.<sup>(20)</sup> In the case of photoblinking, the temperature sensitivity increases with time. In addition to these two phenomena, the rotational correlation time and fluorescence lifetime also play an important role in increasing or decreasing the temperature sensitivity.

## 4.2 Theoretical estimation of temperature sensitivity

To determine the temperature sensitivity of fluorophores based on Eq. (3), the fundamental anisotropy  $r_0$  and molecular volume  $V$  were obtained from the relationship between FA and  $T/\eta$  by curve fitting based on Eq. (2). The fitting results of fluorescein sodium are presented in Fig. 4. Table 1 shows the parameters obtained by the fitting. We determined the temperature sensitivity of FA by substituting the deduced and measured parameters into Eq. (3). Figure 5

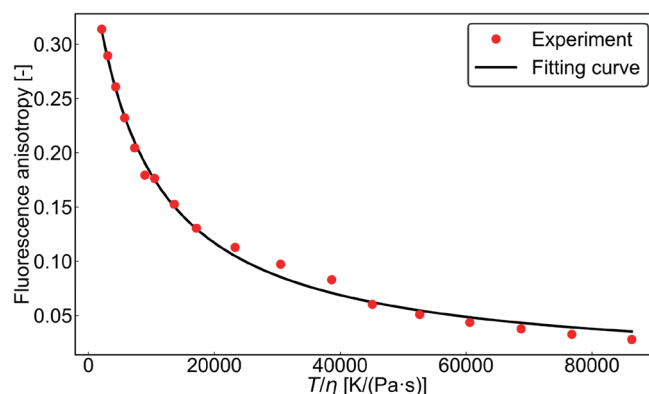


Fig. 4. (Color online) Curve fitting using Eq. (2) to obtain fundamental anisotropy and molecular size of fluorescein sodium solution.

Table 1

Fundamental anisotropy and Stokes radii of fluorescent probe molecules. These parameters were deduced by the curve fitting using Perrin's law.

Probe molecule	Molecular weight (g/mol)	Fundamental anisotropy (-)	Stokes radius (nm)
Fluorescein sodium	376.28	0.389	0.496
	10000	0.360	0.766
FITC–dextran	20000	0.353	0.793
	40000	0.342	0.808
	150000	0.338	0.812

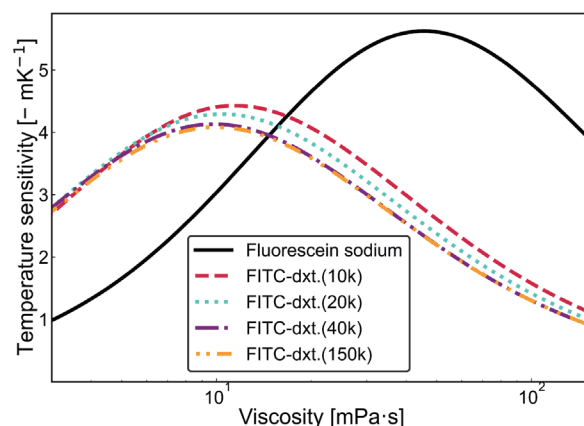


Fig. 5. (Color online) Theoretical temperature sensitivities of FA of fluorescein sodium and FITC-dextran.

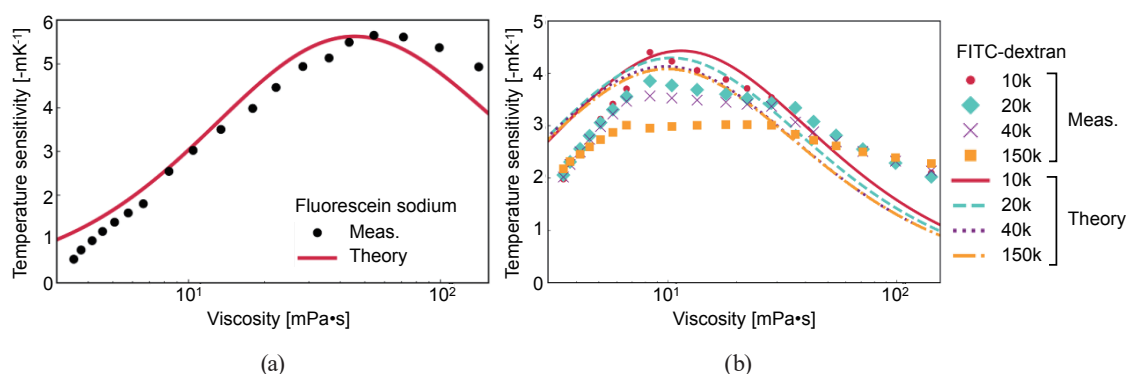


Fig. 6. (Color online) Comparison of theoretical estimation of the temperature sensitivity of FA with experimental results. (a) Fluorescein sodium and (b) FITC-dextran conjugates.

shows the theoretical temperature sensitivities of fluorescein sodium and FITC-dextran. A shift of the peak sensitivity upon labeling, as in Fig. 3, was also found in these results. The increase in the molecular size caused by the labeling slows the rotational motion of the probes, which causes the peak to shift to a lower viscosity. In the FITC-dextran conjugates, the smaller the labeled dextran, the greater the sensitivity. Figure 6 shows a comparison between the theoretical and experimental temperature sensitivities. For fluorescein sodium [Fig. 6(a)], both sensitivities agree well. For the FITC-dextran conjugates [Fig. 6(b)], the overall trends are similar, including the peak viscosity, but the dependence on the molecular weight of dextran only has qualitative agreement. When the literature values of molecular sizes<sup>(21)</sup> were used, the sensitivities were greatly decreased and became one order smaller than our experimental and estimated results. Compared with a previous study,<sup>(22)</sup> our results more accurately describe the temperature sensitivity over a wide range of viscosity. It is also revealed that the contribution of the second term in Eq. (3) becomes one order larger than that of the other two terms. This difference in the contribution is mainly due to the large  $T/\eta$  rather than the temperature-dependent viscosity  $\partial\eta/\partial T$ . The importance of the viscosity term was also implied by Kaur *et al.*,<sup>(18)</sup> and our study quantitatively supports this. The disagreement for the FITC-dextran conjugates may be due to

the complex structure of dextran, which consists of many glucose molecules linked by linear and branched bonds.<sup>(23,24)</sup> Perrin's theory is based on the assumption of the movement of spherical molecules in a viscous medium as an approximation. This assumption might be only partially valid in the case of labeled fluorophores in the present study.

From the above, we can conclude that Perrin's law can be used for the qualitative prediction of the design of thermal probes and that there is no alternative to using an experimental approach to obtain quantitative information of the temperature sensitivity of FA for various molecules.

## 5. Conclusions

In this study, we measured the temperature sensitivity of the FA of fluorescein-based molecules to investigate the effect of solution viscosity on the temperature sensitivity of FA. The results showed that when the sample viscosity was 15.4 mPa·s, fluorescein sodium and FITC–dextran showed higher temperature sensitivity at high and low viscosities, respectively. The temperature sensitivity of FA was compared with the theoretical estimation from Perrin's law. It was confirmed that a viscosity at which the temperature sensitivity reached a maximum value existed, and that theoretical and experimental values were consistent for the fluorescein sodium salt. The contribution of the viscosity term has a greater effect on the temperature sensitivity than the other parameters. It was found that the temperature sensitivity can be adjusted and improved by changing the size of the fluorescent molecules according to the viscosity of the target sample.

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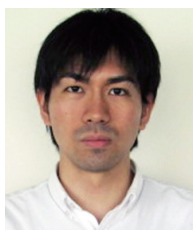
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