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Article

Fluorescence Anisotropy Studies of Fluorescein

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Abstract: Fluorescein anisotropy, which is a widely used technique to study the folding state of proteins or affinity of ligands, is used in the present work to study the temperature sensing of fluid in a microchannel, by adding fluorophore in the fluid. Fluorescein was used as a temperature probe, while glycerol-aq. ammonia was used as a working fluid. Fluorescence anisotropy of fluorescein were measured by varying various parameters. Apart from this, a comparison of fluorescence anisotropy and fluorescence intensity is also performed.

Keywords: Fluorescence Anisotropy; Fluorescein; Glycerol

1. Introduction

With the advancement of micro-and nano-technology, microfluids devices have gained a lot of importance in medical, engineering, and biological fields because of the advantages that, these devices require small sample volume, the reaction rate is fast, and also, they are portable [1]. It is well-known that, thermal transport at micro/nano-scale are different from those at macroscale due to increased surface-to-volume ratio. The thermal behaviour in the microfluidic device is governed by heat conduction rather than convection. In addition, the small heat capacity of the system results in a rapid thermal response. Also, thin liquid film is often used to enhance thermal transport between two solid plates as thermal interface material (TIM). TIM has a nonuniform thickness of the order of micrometers, corresponding to surface roughness of the plates. So, it is necessary to measure the temperature of a fluid in a microchannel [2], as this is key to evaluate performance of polymerase chain reaction (PCR) and potential of novel nanomaterials for hyperthermia [3, 4]. To measure the temperature of fluid in microchannel, microfabricated thermocouples are used [5]. The problem with the thermocouples is that the sensor size is large as compared to the channel size. Also, thermocouples allow us to measure temperature only at the point of contact. Alternative to micro-thermocouples is an optical thermal probe, e.g., laser-induced fluorescence (LIF) [6, 7] using fluorescent molecules and thermochromic liquid crystals [8]. Although LIF is better than micro-thermocouples as LIF is a contact-less technique that relies on thermal quenching of fluorescent molecules [6, 7]. LIF also has drawback like, LIF illumination depends on the concentration distribution of fluorescent probe molecule in the fluid. If the concentration is non-uniform, then the fluorescence illumination is also non-uniform. An alternative to LIF is two-colour LIF [9]. However, two-colour LIF uses an assumption that, there is uniform concentration of two fluorescent dyes in the microfluidic system. In this sense, multiple fluorophore-based thermometry has a disadvantage.

In this present work, we focus on fluorescence anisotropy to measure the temperature of a liquid in microchannel. Many other research groups have used fluorescence anisotropy to measure the folding state of proteins [10]. Fluorescent anisotropy has advantages over LIF, as fluorescence anisotropy is not affected when there is non-uniformity in molecule concentration or incident light intensity because in fluorescence anisotropy, the anisotropy is normalized to the total output light intensity [11].

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Fluorescence Anisotropy: Unpolarized light after passing through a polarizer becomes linearly polarized. This linearly polarized light is allowed to pass through a sample containing fluorophore (i.e., fluorescent dye). Fluorescent molecules have transition dipoles associated with them. The molecules whose transition moment is parallel with respect to incoming polarized light, those molecules get excited to the higher state. The probability that a molecule gets excited is proportional to $\cos^2\theta$ (where θ is the angle between the electric field vector of the incident light and the transition dipole moment of the fluorophore, Fig. 1), and this process is known as "photo-selection". When the excited molecule comes back to the ground state, it emits fluorescence. At the output we measure the parallel ($I_\parallel$) and perpendicular ($I_\perp$) components of the light, where parallel and perpendicular means parallel and perpendicular with respect to the incoming light. If the incoming light is polarized in x-direction, then at the output $I_\parallel = I_x$ and $I_\perp = I_y = I_z$. This is shown in Fig. 2. Mathematically, fluorescence anisotropy (r) is given using [12-14].

$$r = \frac{I_\parallel + I_\perp}{I_x + I_y + I_z} = \frac{I_\parallel + I_\perp}{I_\parallel + 2I_\perp} \qquad (1)$$

So, we can see that r is normalized to the total intensity ($I_x + I_y + I_z$).

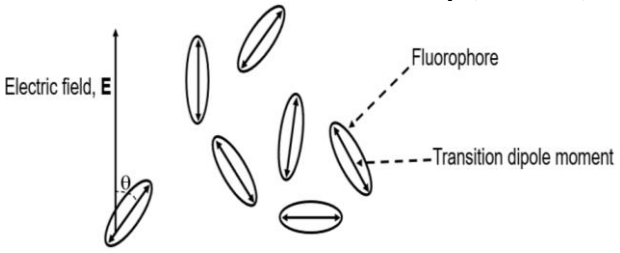

Fig. 1: Schematic representation of photo-selection.

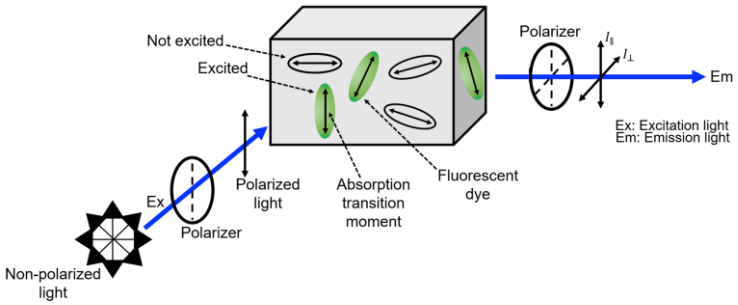

Fig. 2: Schematic of fluorescence anisotropy.

Apart from Eq. (1), we also define Perrins's equation and Stokes-Einstein equation as [15]:

$$\text{Perrin's equation: } \frac{r_0}{r} = 1 + \frac{\tau}{\theta} \qquad (2)$$

$$\text{Stokes-Einstein equation: } \theta = \frac{\eta V}{k_B T} \qquad (3)$$

where, r_0 is the limiting anisotropy (anisotropy without any molecular rotation, also known as freezing anisotropy. This is discussed in details later), τ is fluorescent lifetime, k_B is Boltzmann constant, T is temperature of the solution, V is molecular volume and η is solution viscosity, respectively. Combining Eq. (1) with Eqs. (2) and (3), Perrin's equation can be converted as a function of the fluid temperature:

$$r = \frac{r_0}{1 + \frac{\tau k_B T}{V\eta}} \qquad (4)$$

2. Materials and Methods

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To measure fluorescence anisotropy and fluorescence intensity, the fluorescein was used as a thermal probe in the present work. Fluorescein solution is prepared by dissolving fluorescein in glycerol-aq. ammonia solution. Glycerol is used to control its solution viscosity [16]. As stated above, excited molecule while coming back to the ground state, experiences molecular rotation or energy transfer to the other molecules; Förster resonance energy transfer (FRET). Molecular rotation and FRET are major sources of de-polarization [13, 15, 17]. To avoid these, one solution is to increase the viscosity of the solution. So, glycerol is used. Aq. ammonia solution or ammonium hydroxide is used because of its basicity (pH). Fluorescein shows high fluorescence in basic solutions [18-20].

Fluorescein, glycerol, and aq. ammonia (or ammonia water) were purchased from Wako. Fluorescence anisotropy is measured by two instruments, one is spectrofluorometer (JASCO FP8300) and another is optical fluorescence microscope (NIKON Eclipse TE-2000U). The objective lens in the microscope has magnification of 20x and numerical aperture of 0.45. For anisotropy measurements using spectrofluorometer, a typical cuvette with an optical path of 10 mm was used and no special arrangement was done. But for anisotropy measurements using optical fluorescence microscope, the working fluid was seed in a microchannel fabricated using polydimethylsiloxane (PDMS) by softlithography. The microchannel has nonuniform depth, equivalent to the optical path, around 20 μm to 40 μm.

3. Results and Discussion

As stated in the introduction, fluorescence anisotropy is independent of sample's depth. To confirm this, we measured fluorescence intensity and anisotropy of fluorescein solution in a depth-varying channel, where the depth is varied from 40 to 20 μm. The result is shown in Fig. 3. It can be clearly seen that irrespective of sample's depth, anisotropy is nearly uniform, while intensity varies greatly with channel depth. This is because fluorescence anisotropy is normalized by the total intensity (Eq. 1). Tasumi *et al.* have utilized fluorescence polarization to measure the temperature of a liquid in a microchannel [21]. But, polarization and anisotropy are different in the sense that anisotropy is normalized to the total intensity, whereas polarization is not normalized. Therefore, it can be stated that the fluorescent anisotropy is more versatile parameter in the use of nonuniform situations; rough surface, light illumination intensity and dye concentration.

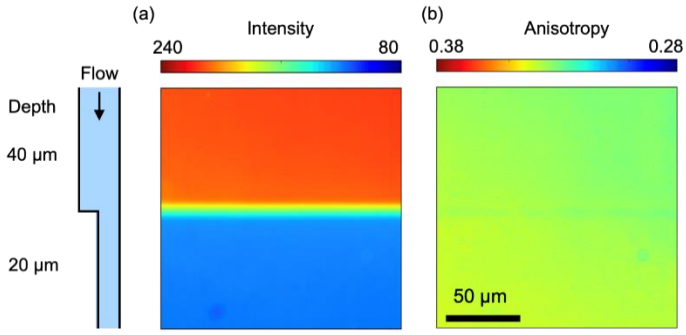

Fig. 3: (a) Fluorescence intensity and (b) fluorescence anisotropy for fluorescein solution in microchannel with different depths.

Figure 4 shows the anisotropy of fluorescein solution by varying the glycerol concentration, measured at 30 and 50 °C. As seen from Fig. 4, as the glycerol percentage is increased i.e., as the solution becomes more and more viscous, anisotropy increases. This is because, as the viscosity of the solution increases, the molecular rotation of the excited molecules, coming back to ground state is reduced by the viscous nature of the solution. Molecular rotation is a source of de-polarization [15].

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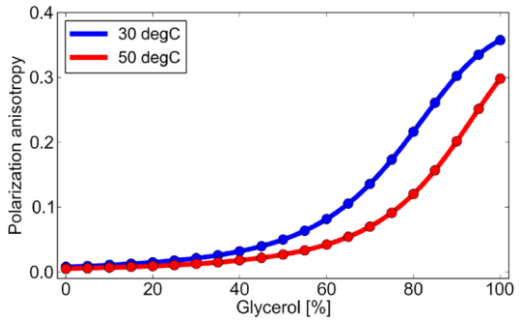

Fig. 4: Fluorescence anisotropy of fluorescein solution as a function of solution's viscosity.

Temperature dependence of fluorescence anisotropy is shown in Fig. 5, where calibration curve up and down means calibration curve obtained in heating and cooling process. As temperature goes from 25 to 65 °C, fluorescence anisotropy decreases. This is because with increase of solution temperature, molecular rotation increases, and as stated above, molecular rotation is a source of fluorescence depolarization. Figure 5 shows that, fluorescence anisotropy showed a negative temperature dependence, and temperature gradient is -0.0045 K^{-1}.

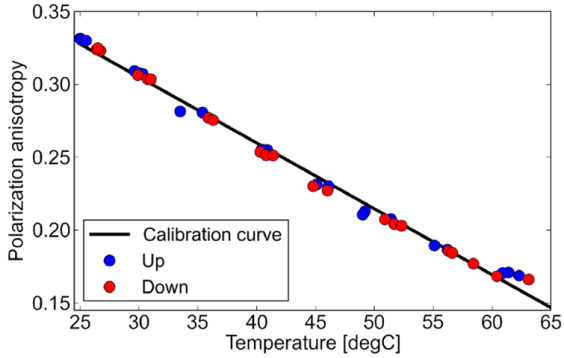

Fig. 5: Fluorescence anisotropy of fluorescein solution as a function of temperature.

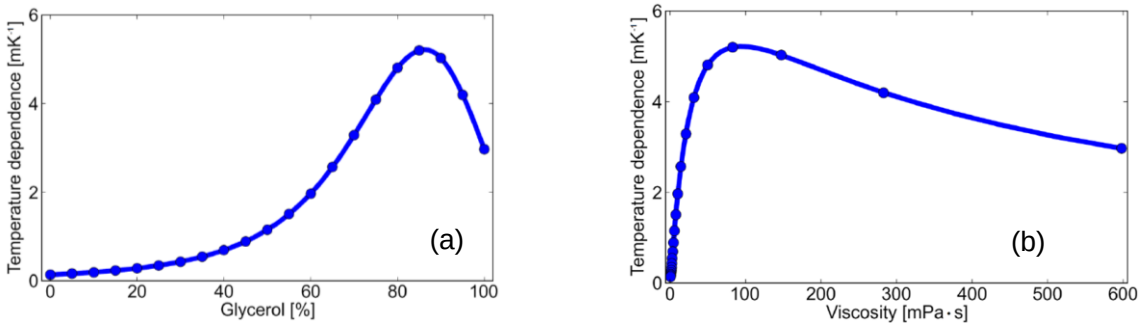

Fig. 6: Temperature dependence of fluorescein as a function of (a) glycerol percentage and (b) viscosity of the solution.

Figure 6 shows the temperature dependence of fluorescein as a function of glycerol percentage and solution viscosity. Figure 6 is just a derivative of Eq. 4 and Fig. 4. It shows that we must select the solution viscosity and temperature wisely, because after a certain viscosity of the solution, the fluorescence temperature dependence starts to show negative trend.

A comparison of anisotropy measured using spectrofluorometer and fluorescence microscope are compared with that of anisotropy values measured theoretically using the

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converted Perrin's equation (Eq. (4)). To calculate anisotropy, theoretically from Perrin's equation, values shown in Table 1 are used, while used temperature was 30 °C (Fig. 7(a)) and 50 °C (Fig. 7(b)). Viscosity is calculated using the method provided by Cheng et al. [22]. It is seen that theoretical value matches well with the calculated value of the measured value from spectrofluorometer. There is some discrepancy between the values obtained by spectrofluorometer and optical system. This is because in optical system, anisotropy is also affected by the collection angle of the fluorescent light, namely the numerical aperture (x_{NA}) of the lens used [23]. Both, 30 and 50 °C measurements show same trend, as shown in Fig. 6(a). Figure 8 shows the temperature dependence of fluorescence anisotropy for theoretical and measured values. It is clearly shown that the effect of optical system on the fluorescent anisotropy is cancelled when we just consider its temperature dependence. This fact helps to establish a simple calibration procedure in the temperature measurement. You just measure the fluorescent anisotropy at a temperature, e.g., room temperature, then can use the theoretical temperature dependence.

Table 1: Physical properties of fluorescein.

Parameter	Value
Limiting anisotropy, r_0	0.38
Fluorescent lifetime, τ	0.37 ns
Volume, V	0.41 nm^3

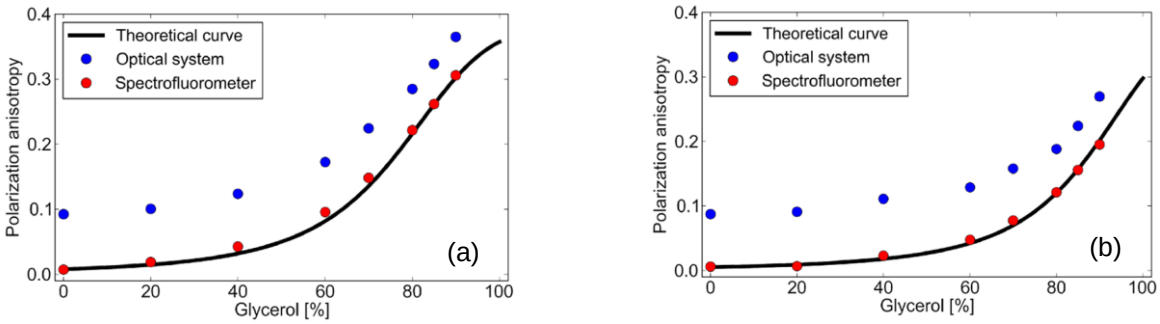

Fig. 7: Theoretical and measured fluorescence anisotropy values at (a) 30 °C and (b) 50 °C.

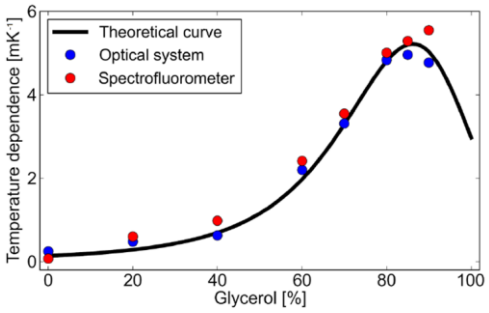

Fig. 8: Temperature dependence of theoretical and measurement value.

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5. Conclusions

Fluorescence anisotropy can be a good candidate to measure the temperature of a fluid in a microscopic domain. As the temperature of the fluid increases from 25 to 65 °C, anisotropy decreases due to the increased molecular rotation. Fluorescence anisotropy shows a negative temperature dependence with a temperature gradient of -0.0045 K^{-1}. As the viscosity of the solution increases, molecular rotation of the excited state molecules reduces, and hence anisotropy increases with a peak. Although the fluorescence

anisotropy is affected by optical system, especially magnification optics, this effect is negligible small in the temperature sensitivity.

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Conflicts of Interest The authors declare no conflict of interest.

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