35th Annual International Conference of the IEEE EMBS
Osaka, Japan, 3 - 7 July, 2013

Toward Reducing Uncertainty in Fluorescence Recovery After Photobleaching*

Jeonghoon Lee, Donghee Lee, Myoung-Ock Cho, and Jung Kyung Kim

Abstract— **We investigate the uncertainty associated with the Fluorescence Recovery After Photobleaching, FRAP, which is widely used in the determination of diffusion coefficient for bio molecules. The uncertainty of our FRAP technique stems from the measurement of the spot size and the half time. The uncertainties of the FRAP is evaluated by considering the uncertainty propagation through the measurements of both spot size and the half time. Finally, we suggest an approach to estimate the effective diffusion coefficient by considering slip conditions between the fluorescent beads and the fluid. The diffusion coefficients measured by the FRAP is close to those obtained from the Stokes-Einstein relation together with the slip correction factor rather than that obtained solely by the Stokes-Einstein equation.**

I. Introduction

Fluorescence Recovery After Photobleaching, so called FRAP, has been utilized as a tool for measuring diffusion for more than 4 decades since Axelrod and his coworkers devised it for the first time in 1976 [1]. Since then, improvement has been achieved by many researchers in various ways. For example, Yguerabide et al. [2] suggested an analytic method to reduce uncertainties stemming from systemic errors. Another improvement in an analysis method was attempted by Feder et al., who included the effects of immobile fraction [3]. From the view point of the lights source, Soumpasis derived a formula close to exact solution for FRAP by employing a uniform circular laser beam [4]. FRAP was also used to reveal the CD2-CD58 interaction in contact areas [5] and binding characteristics of protein to chromatin [6]. Using a specially fabricated FRAP, the diffusion coefficients of fluorescent dextran in porcine articular cartilage was measured [7]. More recently, FRAP was used to analyze the dynamic behavior of the solutes on cell membrane, and it was also applied to in-vivo study such as extracellular space in brain [8] and tumors [9, 10] with fiber optics. Despite long history of the FRAP technique, it is difficult to find how the uncertainty in the measurements is related between the experimental parameters. Motivated by this, we attempt to evaluate the uncertainty associated with the measurement of the spot size and the half time by uncertainty propagation method. In addition, to the best of our knowledge, it is for the first time that the slip condition is considered in the FRAP experiments for the determination of diffusion coefficient.

*This work was supported by the Basic Science Research Program through the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology, Republic of Korea (2010-0016627, 2012R1A1B4002700).

J. Lee is with the School of Mechanical Engineering, Korea University of Technology and Education, Cheonan, 330-708 South Korea (e-mail: jlee@koreatech.ac.kr).

D. Lee was with the Department of Mechanical Engineering, Graduate School, Kookmin University, Seoul 136-702 South Korea. He is now with the Department of Mechanical and Materials Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588 USA (e-mail: hiro0308@hanmail.net)

M.-O. Cho was with the Department of Mechanical Engineering, Graduate School, Kookmin University. She is now with PaSce co. (corresponding author to provide phone: 82-2-910-5409; fax: 82-2-910-4839; e-mail:fallslover@naver.com).

J. K. Kim is with the Department of Family Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria 0084 South Africa, on sabbatical leave from the School of Mechanical Systems Engineering, Kookmin University (e-mail: jkkim@kookmin.ac.kr).

II. Methods

A. Fluorescence Recovery After Photobleaching

Fluorescence recovery after photobleaching (FRAP) denotes an optical technique capable of quantifying the two dimensional lateral diffusion of a molecularly thin film containing fluorescently labeled probes, or to examine single cells. This technique is very useful in biological studies of cell membrane diffusion and protein binding [11].

We can calculate the two dimensional diffusion coefficients assuming the beam profile having a uniform circular shape, which is reasonable [4]. The diffusion coefficient is given by the following equation.

$$D = 0.224 \frac{w^2}{t_{1/2}} \qquad (1)$$

In (1), w is the spot size and $t_{1/2}$ denotes the half time.

B. Uncertainty Propagation

Uncertainty propagation, as a statistics terminology, means the effect of variables' (or measurement parameters') uncertainties (or errors) on the uncertainty of a function based on them. When the variables are the values of experimental measurements they have uncertainties due to measurement limitations (for example, instrument precision, detection limit, etc.) which propagate to the combination of variables in the function [12].

For the FRAP technique, the diffusion coefficient is estimated by measuring the spot size, w, and the half time, $t_{1/2}$, as can be seen in (1). Then the combined uncertainty is associated with the uncertainty in the spot size and the uncertainty in the half time. The combined uncertainty is calculated from (2) [13]

$$U_{D\_FRAP} = \sqrt{\left(\frac{\partial D}{\partial w}U_w\right)^2 + \left(\frac{\partial D}{\partial t_{1/2}}U_{t_{1/2}}\right)^2} \quad (2)$$

, where U indicates the uncertainty and the subscripts imply the measurement parameters.

The combined uncertainty in the diffusion coefficient for the FRAP can be calculated as

$$U_{D\_FRAP} = \sqrt{\left(0.448\frac{w}{t_{1/2}}U_w\right)^2 + \left(-0.224\frac{w^2}{t_{1/2}^2}U_{t_{1/2}}\right)^2} \quad (3)$$

III. Experimental

In our FRAP technique, we prepared solutions of several beads. First of all, 210 nm sized fluorescent beads (FC02F, Bangs Laboratories, Fishers, IN, USA) were prepared. The beads show excitation peak at the wavelength of 480 nm, and emission peak at 520 nm. Next, we prepared 500 nm sized fluorescent beads (Fluosphere F8813, Invitrogen, Carlsbad, CA, USA) which show the excitation peak at the wavelength of 505 nm and the emission peak at 515 nm. The purpose of selection of these two fluorescent beads is to compare the diffusion coefficients for differently sized beads determined by using the FRAP. Concentrations of 210 nm sized fluorescent beads are 0.2 % and those of 500 nm sized fluorescent beads are 0.4 %. To validate the measurement obtained from the FRAP system, we also prepared 70 kDa fluorescein dextran (D1823, Invitrogen, Carlsbad, CA) which showed the excitation peak at the wavelength of 494 nm and the emission peak at 521 nm. Concentration of 70 kDa fluorescein dextran is 2 mg/ml. We attempted to maintain the temperature of the samples at a 25 °C using a heating plate (Live Cell Instrument, HP-R-10, Seoul, Korea) during the measurement of diffusion coefficients in order to minimize the effect of temperature on the determination of diffusion coefficients.

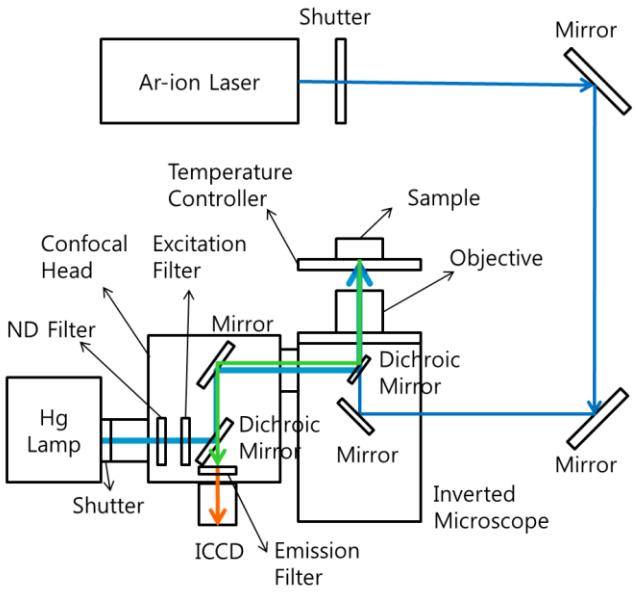

Figure 1. Schematic of the experimental apparatus used in this study

Fig. 1 shows the schematic of our FRAP setup. In the present system, careful attention should be paid to the alignment of the excitation laser beam. The laser beam should be focused near the center of the sample for better image analysis. We used two pinholes to confirm that the Ar-ion laser was propagated parallel to the optical bread board where optical components were installed. We adjusted two mirrors to control the direction of the beam toward the inverted microscope. We made a fine adjustment of the mirrors again, which enabled the laser beam to be aligned on the sample.

We obtained focused images by utilizing an inverted microscope (IX71, Olympus, Tokyo, Japan) equipped with a 60x objective lens (Olympus) and recorded the images of the sample using AQM6, a software (Kinetic Imaging, Nottingham, UK) which allowed us to control the shutter and ICCD camera (Dicam-Pro, Cooke, Romulus, MI). In the FRAP experiments, we bleached the sample for approximately 250 ms with a 488-nm Ar-ion laser (25 mW, 35 LAP 431, Melles Griot, Carlsbad, CA, USA), and the bleaching time was controlled by the shutter located in front of the Ar-ion laser. The fluorescent images were obtained by an ICCD camera at 4 frames per second after photobleaching the sample.

In the measurements, we used specially designed fluorescent beads containing large stokes shift (excitation peak at the wavelength of 488 nm, emission peak at 560 nm in the present study). We captured the time series FRAP images in each frame. By separating the emission wavelength of the fluorescence, we conducted FRAP analyses. In order to detect the fluorescence signal, we used a mercury lamp with a neutral density filter; thus, we could monitor the real time fluorescence image of the FRAP through the AQM6.

IV. Results and Discussion

We measured the diffusion coefficients of fluorescent beads (210 nm, 500 nm) and the fluorescein dextran (70 kDa) in solution by using our FRAP system.

We conducted FRAP experiments for the solution of fluorescent beads (210 nm and 500 nm). During the FRAP experiments, we obtained the time-series images of the samples as well as the fluorescence recovery curves as shown in Fig. 2. Photobleached spot is clearly observed at time = 0 and the bleached spot fades away since it is recovered as time goes by. The fluorescence recovery curves showed that the 210 nm sized fluorescent beads diffused faster than 500 nm sized ones, which implies that our FRAP system is properly operated. (Figures for 500 nm were not shown here.)

The minimum beam spot size of the present study was measured to be 12.6 μm and the maximum of that was 24.5 μm. For the half time, the minimum was measured to be 10.48 s and the maximum was 44.68 s. The uncertainties associated with the beam spot size and the half time are approximately 10%, respectively. Therefore, we can evaluate the uncertainty

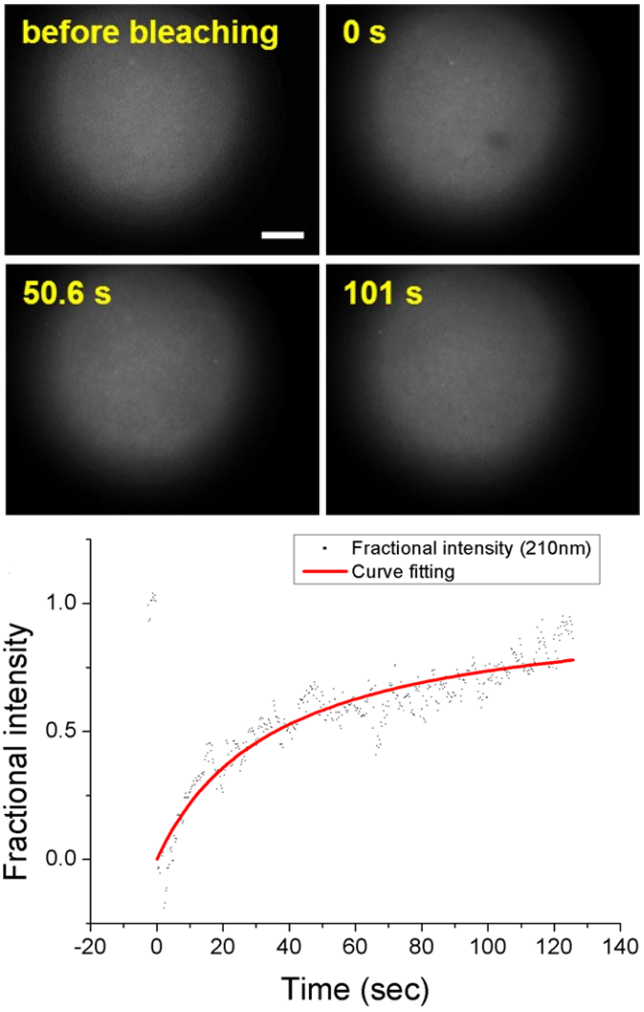

Figure 2. FRAP images of fluorescent beads of 210 nm and the fluorescence recovery curve

propagation due to the beam spot size and the half time using (3). The uncertainty of the FRAP technique is calculated to be from 15.7 to 22.4 %.

The diffusion depends on the particle size and environment surrounding the particle, more specifically, diameter, temperature and viscosity of the fluid. The Stokes-Einstein equation below shows the relationship between the particle size and the diffusion coefficient. We calculated theoretical diffusion coefficients of known sized spherical particles by using Stokes-Einstein equation. Then we compared diffusion coefficients deduced by both experiment and theory.

$$D = \frac{k_B T}{6\pi\eta r} \qquad (4)$$

In (4), k_B is the Boltzmann's constant (1.38×10^{-23} J/K), η is the viscosity, T is the absolute temperature, and r is the radii of the molecules.

TABLE I. DIFFUSION COEFFICIENT MEASURED AND CALCULATED IN DIFFERENT WAYS

	Diffusion coefficients ($\times 10^{-8}$ cm^2/s)		
	FRAP	***Stokes-Einstein equation***	***Slip correction factor considered***
Fluorescent bead (210 nm)	4.10±0.066	2.08	4.29
Fluorescent bead (500 nm)	1.13±0.678	0.87	1.26
Fluorescein dextran (MW = 70 kDa)	12.8±0.019	29.2	N/A

Cunningham correction factor is introduced to consider the effect of slip between the fluorescent bead and the solution. The diffusion coefficient modified using Cunningham correction factor is expressed as

$$D = \frac{k_B T}{6\pi\eta r} C_C \qquad (5)$$

, where C_c denotes the Cunningham correction factor [14]. The Cunningham correction factor in (5) depends on the ratio of the mean free path (λ) of the medium and the diameter (d) of the fluorescent bead as shown below in (6).

$$C_c = 1 + 2.52\frac{\lambda}{d} \qquad (6)$$

Let us assume that the fluorescent bead diffuses within water solution and the fluorescent bead solution is heated by the irradiation of laser beam source. Then, the solution can be locally vaporized by the local heating. When the local temperature of the water solution reaches 75 °C, the saturation pressure is 38.58 kPa. Then, the mean free path can be calculated to be 88 nm from (7).

$$\lambda = \frac{k_B T}{\sqrt{2}\pi d_m^2 P} \qquad (7)$$

In (7), d_m means the molecular diameter and P is the pressure [14]. For the 210 nm fluorescent beads, C_c is calculated to be 2.06 and C_c for 500 nm ones is 1.44. The diffusion coefficients recalculated by considering the Cunningham correction factor are shown in the Table 1. As can be seen, the diffusion coefficients calculated using Cunningham correction factor are close to the values obtained from FRAP experiments within 10 % of the absolute values. This result implies that the sample may be heated up, even though it is instantaneously, due to the local heating by the incident laser beam, resulting in the warmer environment than the initially prepared solution. The local heating seems affect the sample only at the initial state, resulting in the change in

the temperature only at the initial state after which the temperature and the viscosity are independent on time. Of course, the Stokes-Einstein equation holds only for the steady state. Therefore, it is not unreasonable to assume that the viscosity and the temperature of the solution remain unchanged as time goes by in this study. The higher accuracy will allow the diffusion coefficient measured through this correction to be recognized as the more reliable.

V. Conclusion

The uncertainty associated with the FRAP was investigated from the measurement of the spot size and the half time. The uncertainties of the FRAP was evaluated by considering the uncertainty propagation through the measurements of both spot size and the half time. Slip between the medium and the molecules was considered to correct the diffusion coefficient determined solely by the Stokes-Einstein equation. The diffusion coefficients measured by the FRAP was similar to those obtained from the Stokes-Einstein relation together with the slip correction factor rather than that obtained solely by the Stokes-Einstein equation.

References

[1] D. Axelrod, D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb, "Mobility measurement by analysis of fluorescence photobleaching recovery kinetics," *Biophys. J.*, vol. 16, pp. 1055-1069, 1976.

[2] J. Yguerabide, J. A. Schmidt, and E. E. Yguerabide, "Lateral mobility in membranes as detected by fluorescence recovery after photobleaching," *Biophys. J.*, vol. 39, pp. 69-75, 1982.

[3] T. J. Feder, I. Brust-Mascher, J. P. Slattery, B. Baird, and W. W. Webb, "Constrained diffusion or immobile fraction on cell surfaces: a new interpretation," *Biophys. J.*, vol. 70, pp. 2767-2773, 1996.

[4] D. M. Soumpasis, "Theoretical analysis of fluorescence photobleaching recovery experiments," *Biophys. J.*, vol. 41, pp. 95-97, 1983.

[5] M. L. Dustin, "Adhesive bond dynamics in contacts between T lymphocytes and glass-supported planar bilayers reconstituted with the immunoglobulin-related adhesion molecule CD58," *J. Biol. Chem.*, vol. 272, pp. 15782-15788, 1997.

[6] R. D. Phair, S. A. Gorski, and T. Misteli, "Measurement of dynamic protein binding to chromatin in vivo, using photobleaching microscopy," *Methods in Enzymology*, vol. 375, pp. 393-414, 2003.

[7] H. A. Leddy and F. Guilak, "Site-specific molecular diffusion in articular cartilage measured using fluorescence recovery after photobleaching," *Ann. Biomed. Eng.*, vol. 31, pp. 753-760, 2003.

[8] M. C. Papadopoulos, J. K. Kim, and A. S. Verkman, "Extracellular space diffusion in the CNS: anisotropic diffusion measured by elliptical surface photobleaching," *Biophys. J.*, vol. 89, pp. 3660-3668, 2005.

[9] M. Magzoub, S. Jin, and A. S. Verkman, "Enhanced macromolecule diffusion deep in tumors after enzymatic digestion of extracellular matrix collagen and its associated proteoglycan decorin," *FASEB J.*, vol. 22, pp. 276-284, 2008.

[10] J. R. Thiagarajah, J. K. Kim, M. Magzoub, and A. S. Verkman, "Slowed diffusion in tumors revealed by microfiberoptic epifluorescence photobleaching," *Nat. Meth.*, vol. 3, pp. 275-280, 2006.

[11] http://en.wikipedia.org/wiki/Fluorescence_recovery_after_photobleaching

[12] http://en.wikipedia.org/wiki/Propagation_of_uncertainty

[13] H. W. Coleman and W. G. Steele Jr, *Experimentation and uncertainty analysis for engineers*, John Wiley & Sons, New York, 1989.

[14] W. C. Hinds, *Aerosol Technology; Properties, behavior, and measurement of airborne particles*, John Wiley & Sons, New York, 1999.