

Observation of Cell-Size Variation under Environmental Stress by Fluorescence Correlation Spectroscopy without Objective Image Magnification

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Abstract: Fluorescence correlation spectroscopy (FCS) without objective image magnification (without using con-focal microscope) was applied to observe the variation in cell size of *Escherichia coli* (*E. coli*) induced by the anti-cancer agent MitomycinC (MMC). In the system without image magnification followed in this study, the suspension of *E. coli* cells was stirred, and the difference in movement due to the different cell sizes induced by the compulsive solution flow was detected. The addition of 0.1-0.4 $\mu\text{g/L}$ of MMC elongated the *E. coli* cell length from about 3.6 to 7.8 μm . The flow cell (i.d. = about 1 mm) also produced a size-dependent correlation curve. The present system is not based on single molecular FCS but is inexpensive and effective at observing the variation in cell size induced by environmental changes.

Key words: Cell size, fluorescence correlation spectroscopy, environmental stress, GFP, *E. coli*.

1. Introduction

Fluorescence correlation spectroscopy (FCS) has been applied with great success in bio- and medical chemistry ever since Gösch et al. [1] employed a con-focal optical system in 2000. The fluorescence correlation function was given as

$$G(\tau) = \frac{\langle I(t+\tau)I(t) \rangle}{\langle I(t) \rangle^2} \quad (1)$$

where $I(t)$ is the real-time detected signal; τ , a delay variable; and t , the time. The notation $\langle \rangle$ indicates the time average.

In the con-focal system, the observation volume is about 1 fL (10^{-15} L), which is sufficient for determining particle size at the molecular level; this is particularly useful for clarifying the behavior of dyes [2, 3], proteins [4], DNA [5, 6], and nanoparticles such as quantum dots [7]. Recent uses of this system

include expanding the dimensions of observation [8], instrument improvement [9-11], fast (μs -order) dynamics [12] and application to solution chemistry [13].

In the previous studies [14, 15], the ordinary optical configuration for fluorescence was adopted to determine algal sizes. Although the observation volume of the present method is 10^4 times larger than that of the con-focal method, the cell size determination ($> 1 \mu\text{m}$) is convenient, and the system itself is simple. In the con-focal system, size is selected on the basis of Brownian motion. On the other hand, the measurement object has to be moved compulsively due to enlargement of the observation volume. Therefore, in the system, the sample solution is stirred to induce a turbulent flow. Capillary flow was also tested in this study. To measure the average cell size with the present system, the system was applied to observing variations in cell size against chemical stress. The system was proved to be a

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convenient tool for measuring cell size variation under changing environmental conditions.

2. Experiment

The optical measurement system used was shown in Fig. 1 as almost the same as mentioned in Ref. [15]. A 20-mW argon laser (488 nm: Spectra-Physics, model 161C-010) was used as the light source, and the output diameter was controlled by a 200- μm pinhole. The movement of the *E. coli* suspension was induced by a stirrer at the bottom of the quartz cell. The fluorescent signal through a pinhole (10 μm) was detected by a photon counter (Hamamatsu, model C8855) with a 490-nm low-cut band-pass filter (Raman) and a 510-nm band-pass filter. The observation volume was about 8×10^{-12} L (about 10^4 times larger than that of the con-focal system). The optical signal was processed by a real-time correlator (ALV-multiple tau digital real correlator 5000/60XO; ALV GmbH Germany), in which the diffusion time was calculated after curve-fitting, and the 3-D diffusion mode was based on the software attached to the ALV correlator. The bead standards (Fluoresbrite microspheres: POL-YG series) were purchased from Polyscience Inc.

The transformation and expression of GFP (green fluorescent protein) were as follows: *E. coli* DH5 α

was contacted with a GFP plasmid (pZs green vector purchased from Clontech Laboratories, Inc., #632446) and was left for 30 min on ice. This *E. coli* was resistant to ampicillin (antibiotic agent) and pre-incubated in the LB (Lythogeny Broth) medium with ampicillin (purchased from Sigma-Aldrich Co.) at a concentration of 0.1 $\mu\text{g}/\text{mL}$ for 2 h. The *E. coli* (30 μL) was incubated with 3 mL of LB medium having different concentrations of MitomycinC (MMC; anti-cancer agent purchased from Kyowa Hakko Bio Inc.) at 37 $^{\circ}\text{C}$ for 3 h and transferred to a PBS solution, after which FCS was performed.

Fluorescence imaging for *E. coli* cells was performed with an incident-light fluorescence microscope (AXIO Imager. A1, Carl Zeiss MicroImaging GmbH, Germany) equipped with $\times 40$ objection lens. Fluorescent proteins of the cells excited by Xenon-Arc-Lamp through a 470 ± 20 nm band-path filter and a dichroic mirror with cut-on wavelength at 495 nm were detected by a digital steel camera (EOS kiss X3, Canon Inc., Japan) through a 525 ± 25 nm band-path filter and $\times 1.6$ photo relay lens. The photo-images were processed with the ImageJ software.

3. Results and Discussion

3.1 Observation of *E. coli* Cell Elongation Induced by MMC in Turbulent Flow

Fig. 2 shows the calibration curve for the diffusion time of the spherical standard beads; measurements were taken 5 times before and after the measurement of *E. coli*. In the system, the diffusion time responded to variations in bead diameter of 1-10 μm . Fig. 3 shows the dependence of the diffusion time on the rotation speed of the stirrer (spinner) in the range from 100 to 300 rpm. An increase in the rotation speed of the stirrer clearly led to a linear decrease in the diffusion time. When the rotation speed exceeded 300 rpm in the system, a correlation curve could not be obtained. In the regular measurement, the rotation speed of the stirrer was set at 300 rpm.

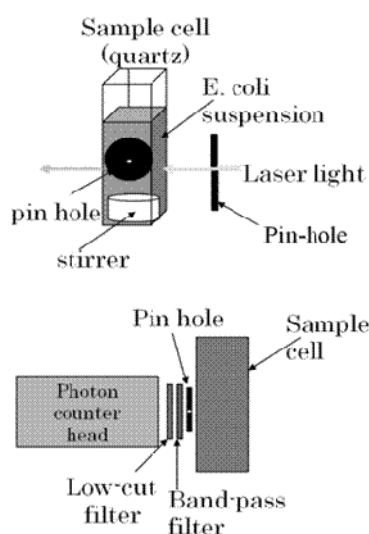


Fig. 1 Optical configuration adopted in this experiment.

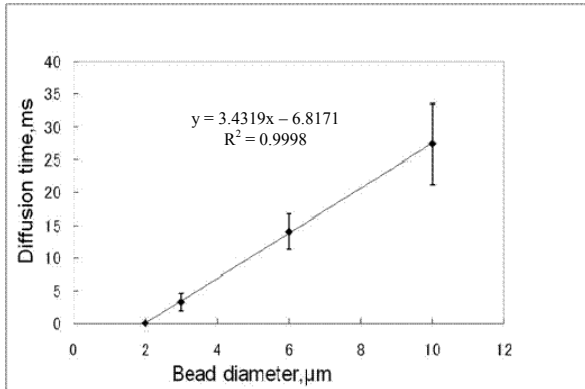


Fig. 2 Calibration for the standard fluorescent beads in the present method.

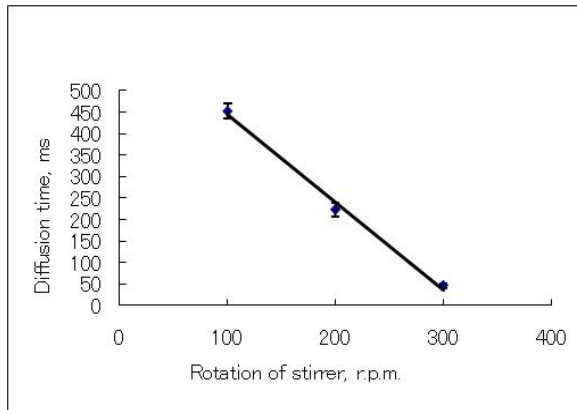


Fig. 3 Dependence of diffusion time of the 10 μm bead on the rotation speed of stirrer.

Fig. 4 shows a fluorescence microscope image of *E. coli* with and without MMC. The elongation of *E. coli* cells induced by MMC could be observed under an optical microscope. As can be seen in the figure, the various sizes of *E. coli* cells exist. Due to our previous work [15], the obtained diffusion time is the average against the cells of various sizes.

MMC was clearly seen to affect the elongation of *E. coli* cells under the microscope. Result of FCS was

shown in Fig. 5. The diffusion time was about 0.55 ms for *E. coli* (for addition of MMC less than 0.1 $\mu\text{g/L}$) and about 8.9 ms for *E. coli* (for addition of MMC at 0.4 $\mu\text{g/mL}$). Addition of MMC is effective for elongation of *E. coli* in the rather narrow range between 0.1 and 0.4 $\mu\text{g/L}$.

In this experiment, spherical fluorescent standard beads were used. However, as shown in Fig. 4, *E. coli* takes a rod-shape. It is pointed out that the rod type body gives square larger in the length given from the diffusion time of the sphere body in the con-focal FCS, in which the particles moves based on the Brownian movement [16]. On the other hand, under the laminar flow, the size discrimination due to its volume is impossible because all the particles flow in the same direction at the same flow rate. Unfortunately, the size standard for rod-type is not commercially available.

According to the above situation, the following scheme was constructed in the case. When the observed particle moves along one direction, the movement equation can be expressed as

$$m\dot{v} = (\rho - \rho_w)Vg + f(U - v) \quad (2)$$

where ρ , ρ_w , and g are the density of the observed particle (standard or *E. coli* cell), density of water, and gravity constant, respectively. f is the resistance constant.

For a standard bead, f is given as

$$f = 6\pi\eta a \quad (3)$$

For an *E. coli* cell, it is

$$f = \frac{6\pi\eta c}{\sqrt{3} \ln(7 + 4\sqrt{3})} \quad (4)$$

In the above case, the shape of the *E. coli* cell is

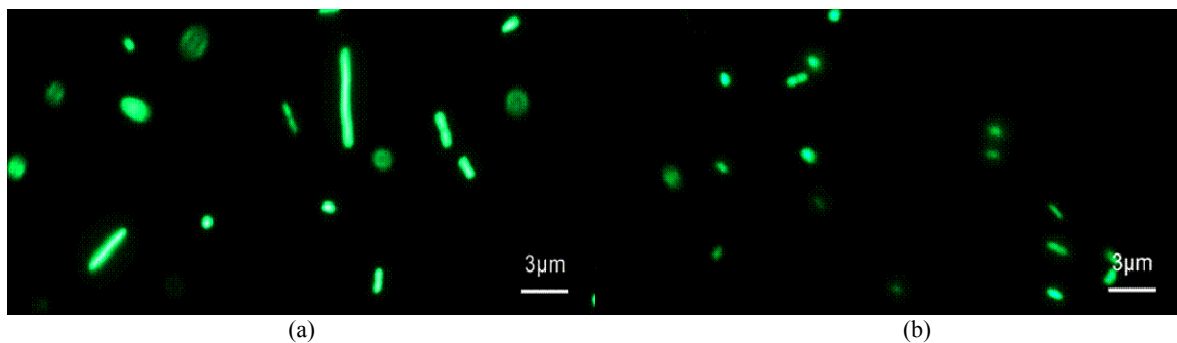


Fig. 4 (a) Microscopic image of *E. coli* with 0.1 $\mu\text{g/mL}$ of MMC; (b) Microscopic image of *E. coli* without MMC.

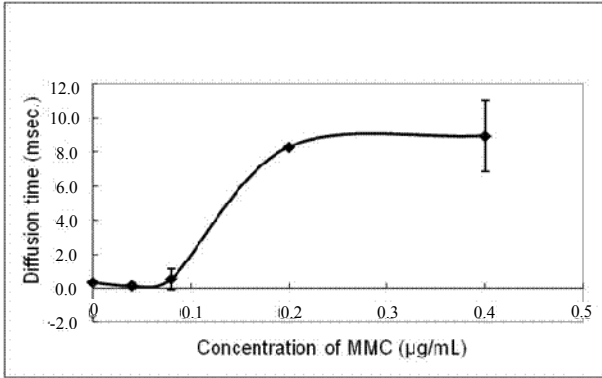


Fig. 5 Diffusion time vs. concentrations of MMC under *E. coli* was grown.

supposed to be an ellipsoid with major and minor axes of $2c$ and c , respectively, and η is the viscosity of water. The steady state (terminal) speed (v) of the objective particle is given by

$$v = \frac{1}{f}(\rho - \rho_w)Vg + U \quad (5)$$

where V is the flow of the water. Here, the step width of the particle in the random-walk δ against the unit time Δt determines the diffusion time (D) in the one-way water flow system.

$$D = \frac{\delta^2}{2\Delta t} \quad (6)$$

The Einstein-Stokes equation also gives D :

$$D = \frac{k_B T}{f} \quad (7)$$

where T is the temperature of the experimental system (293 K) and k_B is Boltzmann's constant. D calculated from Eq. (2), i.e., δ , was obtained. The correlation curve was obtained after varying Δt at increments of

0.01 ms. This method was described elsewhere [18].

Since the diffusion time is at about 0.5 of the correlation function ($G(\tau)$) value, the enlarged part of the correlation curve is given in Figs. 6a and 6b. The calculated results show that the variation in diffusion time against the size variation for the 1:2 ellipsoid (the resemblance for *E. coli*) was larger than that of the spherical particle (the resemblance for the size-standard bead); i.e., when the minor radius of *E. coli* changed from 1 to 2 μm , the time (at $I = 0.5$) increased more than 0.12 ms (0.12 ms increase per 1 μm increase). On the other hand, when the radius of the size standard changed from 0.875 to 5 μm , the time increased by 0.41 ms (0.10 ms increase per 1 μm increase). This means that the difference in volume reflects about 20% increase in the diffusion time of the ellipsoid compared with that of the spherical body.

If it is possible to extend the above consumption to 3-dimensions, the rod length is 1.7 times ($= (1.2)^3$) larger than the radius of the spherical standard. From the size calibration of *E. coli* based on the diffusion time for the spherical bead (size-standard), the diffusion time of 0.55 ms at 0.1 μM of MMC was 2.1 μm and 8.95 ms at 0.4 μM of MMC was 4.6 μm . It can be deduced that the length of *E. coli* can be calculated as 3.6 (2.1×1.7) μm to 7.8 (4.6×1.7) μm when the concentration of MMC increased from 0.1 to 0.4 $\mu\text{g/mL}$.

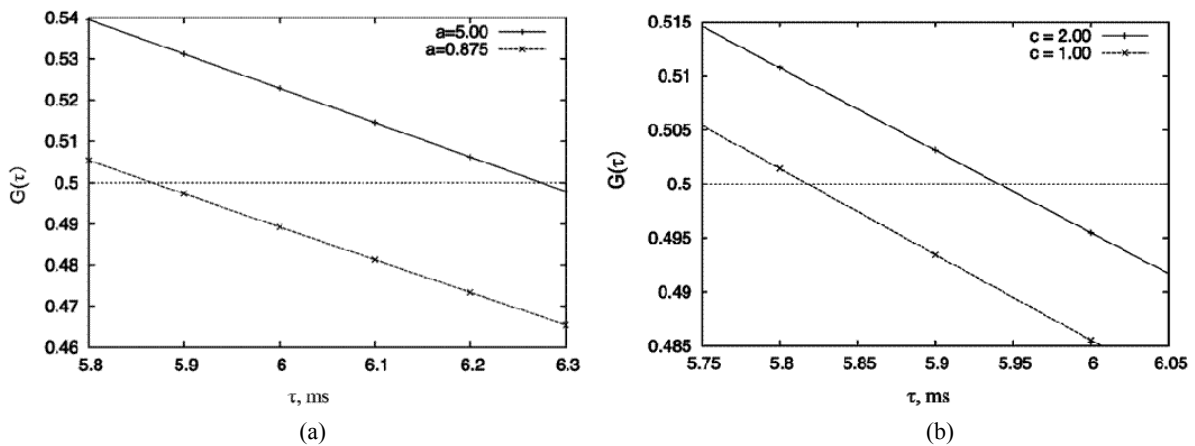


Fig. 6 (a) The correlation curve ($G(\tau)$) for the spherical particle, which are enlarged at ($G(\tau) = 0.5$). a: radius, μm ; (b) the correlation curve ($G(\tau)$) for the ellipse particle, which are enlarged at ($G(\tau) = 0.5$). c: short radius (long radius is $2c$), μm .

3.2 Application to the Capillary Flow System

Fig. 7 shows the FCS observation system for the particle flow. A Teflon tube (AF 2400: transparent in the UV region) with an i.d. of 0.76 mm and o.d. of 1.03 mm was purchased from Random Technologies Inc. (San Francisco, USA). This tube was set vertically in front of the photon counter, and the suspension of standard particles was driven by a syringe pump (Shimano Kenshi Co. Ltd. Japan, model STP-570307-06) controlled by a PC through RS232c. The flow rate of the bead suspension was 0.05 mL/min, and the flow direction was from lower to upper (direction against gravity).

An example of the correlation curves is shown in Fig. 8 obtained from the diffusion time in Fig. 7. The calibration curve (diameter of standard bead vs. diffusion time) of this system is also shown in Fig. 9, as well as the same examples of the correlation curve. The flow rate is the important factor for obtaining the correlation curve. A flow rate higher than 0.7 mL/min did not provide the correlation curves. In addition, a lower flow rate means a longer measurement time. It is important that the size variation can be measurable with this flow system. The narrow i.d. tubes (0.195 and 0.114 mm) currently do not provide a correlation curve; this might be due to the fast flow in these tubes. In terms of practicality, the present flow system for observing the correlation curve is inconvenient compared to the above stirring system. Changing the sample takes about 5 min

(including the time for washing the flow line). In addition, experimental conditions such as the flow rate of the sample and the i.d. of the observation tube are limited in order to obtain the correlation curve. To improve sample introduction, a flow injection technique might be the solution to the capillary flow method.

It inspected the FCS without image magnification. Orthodox FCS is based on the con-focal system, and it has contributed to elucidating the size variations of various molecules [17]. However, an ordinary con-focal FCS system costs over 10 million yen. The paper demonstrates that a method without the con-focal system is valid for measuring the cell size variation under different environmental factors. When a laser diode is applied, the instrumental cost is less than one-tenth that of the present con-focal FCS method. Although the size differentiation under compulsive hydro-flow is essential in the present system, the present method is adequate for measuring micrometer-level size variations and has a high potential for observing biological cell size changes induced by external or internal environmental changes. Orthodox FCS is mainly used for molecular-level detection, and the theoretical fundamentals have been established. Meanwhile, the present method is a compromise and not used for single-molecule detection. However, it is simple, inexpensive, and very practical, especially for the variation in cell size under the environmental stress.

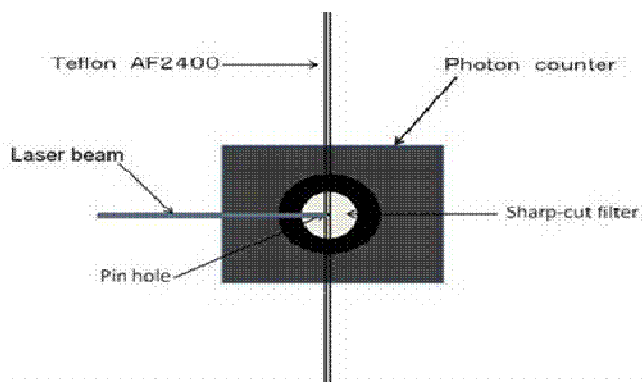


Fig. 7 The front view of the flow system.

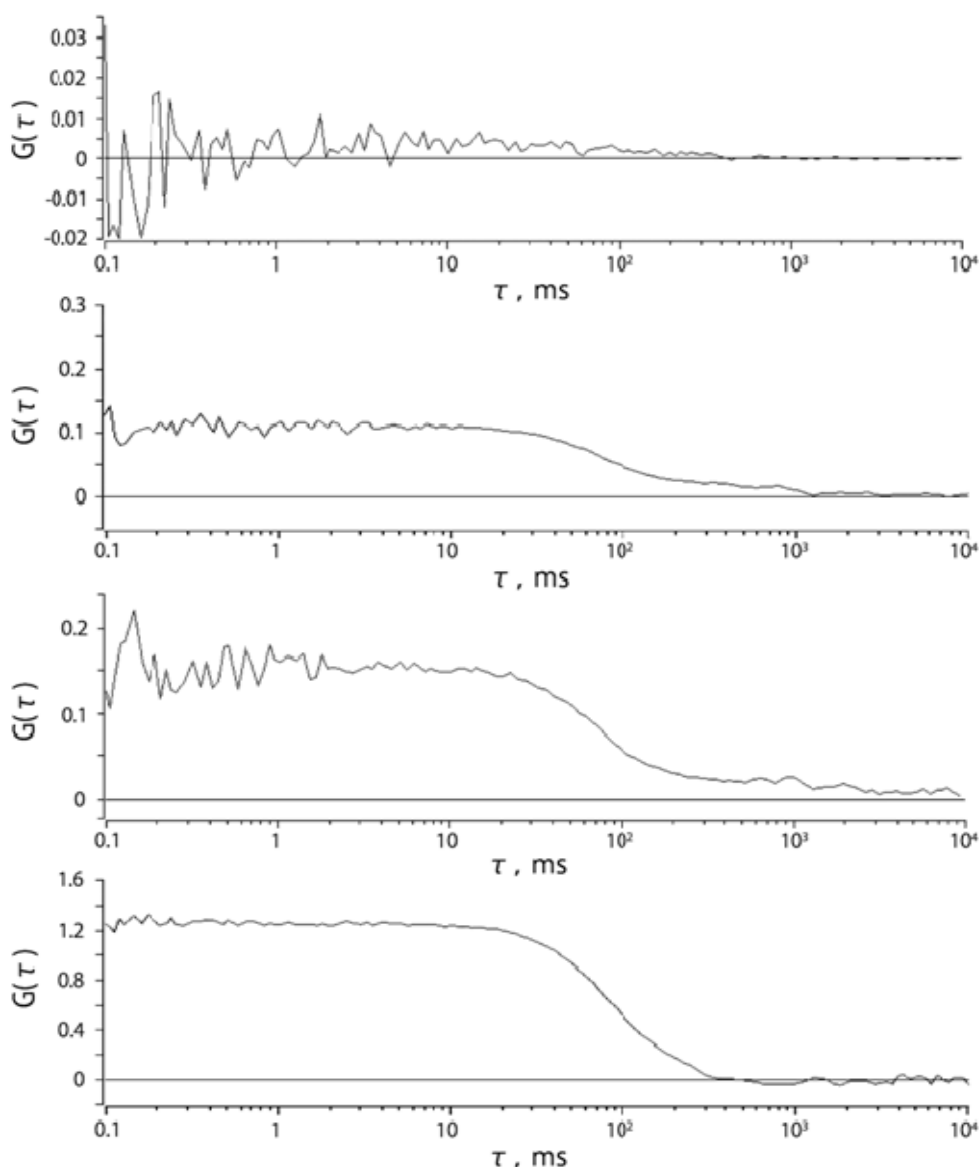


Fig. 8 Examples of correlation curves observed by the system mentioned in Fig. 7. The bead diameters from the upper to lower figures are 1.75, 4, 6, and 10 μm , respectively.

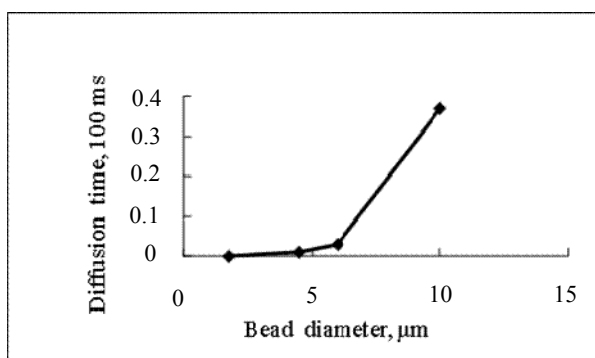


Fig. 9 Observed calibration curve for diffusion time vs. the standard bead diameter. This figure was obtained by using the system shown in Fig. 7.

4. Conclusions

FCS with using con-focal systems has been widely developed in the field of bio or physical chemistry as the single molecular detection. On the other hand, the method proposed in this paper does not employ any image magnification tools. However, this method is simple, inexpensive and applicable for measuring the biological cell of which size is over 1 μm as previously mentioned [15]. In this point, it is the effective method to evaluate the magnitude of the environmental stress to microbes.

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