In-cell Viscosity Measurement Using a Fluorescence Up-conversion Microscope

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Using a femtosecond fluorescence up-conversion microscope, viscosity values in yeast cells (budding, wine, and fission) were estimated based on ultrafast fluorescence dynamics of a fluorescent probe dye, Malachite Green. The decay time of the S_1 state of Malachite Green in cytoplasm was determined to be 2.2–2.4 ps at room temperature, which corresponded to the viscosity value of 5–6 cP. Nondestructive estimation of viscosity value in cells (in-cell viscosity) was carried out.

Innovations of ultrafast lasers have made it possible to investigate ultrafast molecular dynamics, such as electronic and vibrational relaxation processes. Although ultrafast spectroscopic measurement has been conducted mainly in homogeneous systems, its combination with microscopy is important for the investigation of inhomogeneous systems. Recently, we developed two types of ultrafast time-resolved fluorescence microscopes, and femtosecond and picosecond time-resolved fluorescence measurements of microscopic samples were realized.[1–4] In this study, the femtosecond fluorescence up-conversion microscope was used to detect fluorescence in biological samples. In-cell viscosity values were estimated based on fluorescence dynamics of a fluorescent probe dye, Malachite Green (MG). MG is a triphenylmethane (TPM) dye whose relaxation kinetics have been well investigated.[5–11] It is known that the relaxation lifetime of the S_1 state increases with increasing solvent viscosity; it shows $\eta^{2/3}$ dependence due to nonradiative decay because of the viscosity-dependent rotation of the phenyl rings of MG.[5–7] In this letter, we report an attempt of nondestructive estimation of viscosity in cells by measuring ultrafast fluorescence dynamics of embedded MG molecules.

The femtosecond fluorescence up-conversion microscope has been described in detail elsewhere.[1–3] The second harmonic of a Ti:sapphire laser was used as excitation light (400 nm, <12 pJ). The time and spatial resolution of this system were 550 fs for 670 nm fluorescence and $\approx$340 nm, respectively. Yeast strains used in this study were IAM4274 (wine yeast), s288c (budding yeast), and K131 (fission yeast). All yeast strains were cultured in yeast extract (YE) at 30 °C. To prepare the yeast cell extract, 50 mL of culture was centrifuged, and collected cells were washed twice with cold water. The yeast cells were mixed with an equal volume of water and 0.6 mL of zirconium beads. Then, they were disrupted with a multibeads shocker cell disrupter. Less than 20 μL of MG/water solution (10^{-2} M) was mixed with $\approx$1 mL of yeast cells or their extracts. One drop of dyed cells or extract was pipetted onto a cover slip. This was softly pressed with another cover slip to form a thin film between the cover slips. The rim of the cover slips was sealed to prevent evaporation.

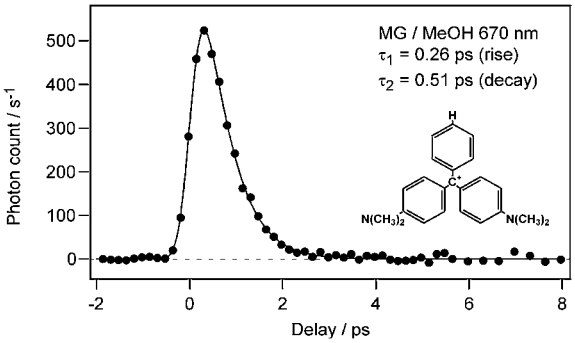

Figure 1. Femtosecond time-resolved fluorescence of MG/MeOH solution observed at 670 nm.

Upon photoexcitation with a 400-nm light, MG molecules were photoexcited to the S_2 state and time-resolved fluorescence from the S_1 state was monitored at 670 nm (the S_1 fluorescence). Femtosecond time-resolved fluorescence of MG/MeOH solution is shown in Figure 1. As has been already reported in detail,[9] the observed time-resolved fluorescence can be reproduced by two exponential functions with lifetimes of $\tau_1 = 0.26$ ps (rise) and $\tau_2 = 0.51$ ps (decay). The solid curve is a fitting result with double exponential functions considering the time resolution of this system (550 fs). The rise component of τ_1 can be assigned to population increase of the S_1 state due to $S_2 \rightarrow S_1$ relaxation, whereas the decay component of τ_2 can be assigned to $S_1 \rightarrow S_x$ relaxation. The S_x state is considered to be a nonfluorescent electronic state having large configuration difference from the ground state or a vibrationally hot ground state. The τ_2 lifetime due to $S_1 \rightarrow S_x$ relaxation increases as the solvent viscosity is changed, following an approximately $\eta^{2/3}$ dependence.[7–9] Using MG molecules as a fluorescent probe, viscosity values in yeast cells were estimated. The femtosecond time-resolved fluorescence from yeast cell extracts stained with MG/water solution was first measured, and the results are summarized in Table 1. Because of the low signal-to-noise ratio, the rise component observed in MG/MeOH solution could not be clearly identified in all samples. However, it was found that the fluorescence decays were reproduced not with a single exponential decay but with two exponential decays. Since the short-lifetime components were $\approx$3 ps for all samples and these values were almost of the same order as the $S_1 \rightarrow S_x$ relaxation lifetime in solution,[10] they can be safely assigned to the $S_1 \rightarrow S_x$ relaxation lifetime. However, the newly appearing long-lifetime component showed a large difference among the samples. According to a previous femtosecond time-resolved absorption study with S_2 photoexcitation, the lifetime of the S_x state was 3 ps[8] for MG/water solution, whereas the $S_1 \rightarrow S_x$ relaxation lifetime was 0.63 ps. The

Table 1. Time constants of fluorescence decay[a]

Yeast	Extracts/ps		In Cell/ps	
budding	2.8 ± 0.3 (38)	46.2 ± 3.1 (62)	2.2 ± 0.4 (66)	25.3 ± 4.9 (34)
wine	2.5 ± 0.4 (25)	93.1 ± 9.0 (75)	2.4 ± 0.2 (62)	27.6 ± 4.4 (38)
fission	3.2 ± 0.2 (57)	33.5 ± 2.4 (43)	2.4 ± 0.2 (63)	26.3 ± 5.9 (37)

[a]The values in parentheses are the percentage of amplitudes.

$S_x \rightarrow S_0$ relaxation cannot be observed by time-resolved fluorescence spectroscopy because of the large configuration change in the S_x state compared to the ground state. Assuming that the rotational motion of the phenyl rings of MG was hindered by crushed septum or organelles in the cell extract and that the conformation change was not sufficient during the $S_1 \rightarrow S_x$ relaxation, the $S_x \rightarrow S_0$ relaxation would be observable even in the fluorescence measurements. However, the long-lifetime components measured in cell extracts are too slow (i.e., 93.1 ps for wine yeast) compared with that observed in time-resolved absorption spectroscopy. Furthermore, we cannot explain why the lifetime of the S_x state varied so much for each extract while the $S_1 \rightarrow S_x$ relaxation lifetime showed little difference. The central carbon atom of the MG molecule has a positive charge in solution while such molecules as nucleotides, phospholipids, and polyphosphates have negative charge because of the presence of phosphoric acid. Therefore, it is possible that MG molecules bound to such charged molecules gave the slowly decaying component, whereas MG molecules dissolved in cytoplasm showed ≈3 ps dynamics because of the $S_1 \rightarrow S_x$ relaxation. In the preparation of cell extract, we added water and zirconium beads into the yeast culture for disruption. Therefore, even the short decay components of cell extracts cannot reflect the real viscosity value of cytoplasm. The femtosecond fluorescence up-conversion microscope has high spatial resolution, enabling us to conduct direct time-resolved fluorescence measurements of each dyed yeast cell. The femtosecond time-resolved fluorescence observed in yeast cells is shown in Figure 2. We measured the time-resolved fluorescence trace only once from the center of one cell body (not in the schizogenetic motion), and the results shown in Figure 2 are an average of ten cell bodies. The time-resolved intensity measured from yeast cells was very weak because yeasts could not be stained easily with MG. Since the signal-to-noise ratio was much lower than that of cell extracts, the temporal fluorescence rise due to $S_2 \rightarrow S_1$ relaxation also could not be identified. However, the obtained fluorescence traces also had two exponential decays, and the observed lifetime values were almost the same for all yeasts. Obtained results are also summarized in Table 1. As in the case of cell extracts, the short lifetime component having a lifetime of 2.2 to 2.4 ps can be assigned $S_1 \rightarrow S_x$ relaxation. According to previous literature, the $S_1 \rightarrow S_x$ relaxation lifetime can be related to the viscosity value of solvent surrounding MG molecules. At room temperature, for instance, the relationship between $S_1 \rightarrow S_x$ relaxation lifetime and viscosity value is as follows: ≈0.5 ps for 0.6 cP (MeOH), ≈0.6 ps for 1 cP (water), ≈2 ps for 5 cP (mixture of water and glycerol), and ≈5 ps for 21 cP (ethylene glycol).[10] Therefore, the $S_1 \rightarrow S_x$ relaxation lifetime of 2.2 to 2.4 ps observed in the experiments can be related to the in-cell viscosity value and it was determined to be 5–6 cP. Compared with the viscosity of water at room temperature of 1 cP, it was found that the viscosity value of yeast cells was almost 5–6 times larger. In studies

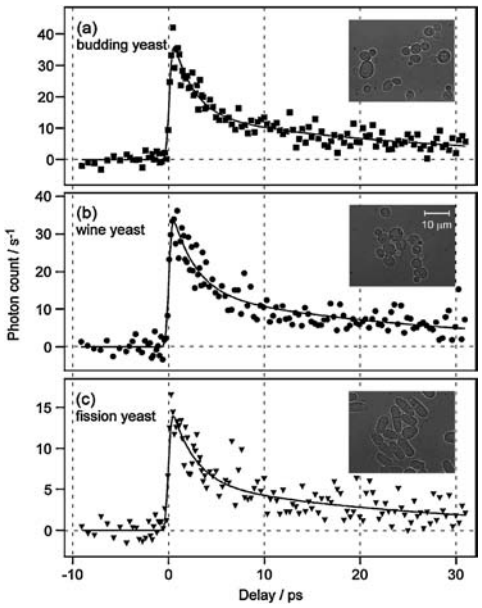

Figure 2. Femtosecond time-resolved fluorescence (670 nm) observed at the center of yeast cells.

of in-cell protein mobility using such techniques as NMR, the viscosity value inside a cell is considered to be important for considering the mobility of proteins in vivo. In such studies, in-cell viscosity has been estimated to be two times larger than that of water.[12] The fluorescence up-conversion microscope has high spatial resolution, so that the evaluated viscosity value may reflect spatial distribution of in-cell viscosity whereas the averaged value could be obtained from the NMR study. Therefore, we consider that this technique enables us to image the in-cell viscosity distribution based on ultrafast fluorescence dynamics. The present work demonstrated a possibility of using femtosecond up-conversion microscopy as a tool to monitor in-cell viscosity of biological samples.

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