SI Text

SI Results. The signals obtained by the developed device by flowing glutathione-labeled Alexa532 at the single-molecule level can be assigned to fluorescence from single fluorophores (Fig. 2). However, there is a possibility that the signals might be caused by a light scattering of impurity particles. To eliminate the latter possibility and to demonstrate that the observed signals were fluorescence, the laser power dependence of the observed signals was investigated. The obtained results could be analyzed consistently by assuming a photocycle of the fluorophore involving the ground state (S_0), the excited singlet state (S_1), and the excited triplet state (T_1). The results therefore demonstrate that the signals are fluorescence from single molecules. Furthermore, the data show that the appearance of T_1 in the photocycle is partly responsible for the fluorescence intensity fluctuation of G-Alexa.

We investigated the dependency of the signal intensity obtained by flowing G-Alexa at the single molecule level on the power of excitation laser. If the signals originate from the impurity particles, the scattered light should be linearly dependent on the excitation laser power. In contrast, if the signals are fluorescence, the intensity should saturate at the higher excitation laser power due to the changes in the rate-limiting step in the photocycle. Under the assumption that the three states, S_0, S_1 and T_1, are involved in the photocycle of the fluorophore, the measurable fluorescence intensity, I_f, at constant excitation can be described by Eq. **1**:

$$I_f \propto \frac{k_S k_T \sigma_{dye} I_{ex}}{(k_S + k_{isc})k_T + (k_T + k_{isc})\sigma_{dye} I_{ex}} \qquad \textbf{[1]}$$

where I_{ex} is the photon flux of the excitation laser, σ_{dye} is the molecular absorption cross section of the dye and is 3.1×10^{-16} cm^2 for Alexa532 at 532 nm, k_S is the rate constant for the sum of radiative and nonradiative transitions from S_1 to S_0, k_T is the rate constant of triplet state depletion, and k_{isc} is the rate constant of intersystem crossing from S_1 to T_1 (1). At the limit of weak laser power, I_f is proportional to I_{ex}, because the second term of the

denominator is negligible. In this condition, the rate-limiting step is the excitation step from S_0 to S_1. In contrast, at the limit of strong laser power, I_f becomes constant because the first term of the denominator is negligible. This corresponds to the changes in the rate limiting step in the photocycle. If $k_{isc} > k_T$, the decay of T_1 is rate-limiting. If $k_{isc} < k_T$, the decay of S_1 becomes rate-limiting. In either case, the saturation of the observed signals demonstrates that the signals are fluorescence.

The laser power dependence of the signals obtained by flowing G-Alexa at the single molecule level is shown in SI Fig. 6. The background intensity increased monotonically upon the increase of laser power, because the background is caused mainly by Raman scattering of water (1, 2). In contrast, the observed signal intensity saturated at ≈200 mW (corresponding photon flux is $\approx 0.4 \times 10^{27}$ $m^{-2} \cdot s^{-1}$), suggesting that the rate-limiting step in the photocycle changes from the excitation step of S_0 to the decay step of either S_1 or T_1 (3). The saturation was analyzed by Eq. **1**. We assumed that k_{isc} is 1.1×10^6 s^{-1}, which is the literature value for Rhodamin 6G (4). The fitting of the data by Eq. **1** gives a k_T value of 3×10^4 s^{-1} and a k_S value of 2×10^8 s^{-1}, respectively. Despite the uncertainty of the excitation photon flux, the estimated k_S value of 2×10^8 s^{-1}, corresponding to a fluorescence lifetime of ≈5 ns, is reasonable for organic fluorophores. Although no value in the literature was reported for k_T of Alexa532 at room temperature, the value for Rhodamin 6G was 1.10×10^6 s^{-1} (4). Considering the uncertainly of excitation photon flux and other parameters, the two values should be considered comparable, and they support the analysis based on the three-state model. The results demonstrate that the observed signals are not caused by a scattering of small particles but by fluorescence from Alexa532. Because $k_{isc} > k_T$, the analyzed data further suggest that the decay of T_1 likely limits the rate of the photocycle.

To confirm that the decay of T_1 limits the rate of the photocycle of Alexa532, the effect of a triplet state quencher on the fluorescence intensity was investigated. We used 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), which was reported to quench the triplet sate of Cy5 (5). To make the experiment easy, the fluorescence intensity was observed at the bulk condition by flowing the samples at relatively high concentration (83.4 pM) to the same flow cell. The laser excitation and fluorescence detection systems

were the same as those used for the single-molecule measurements. The laser power dependence of bulk fluorescence intensity was measured for G-Alexa in the presence of 4 M Gdm with and without 2 mM Trolox (SI Fig. 7). The same experiment was also conducted for cyt *c*-Alexa in the presence of 1 M Gdm (SI Fig. 7). Firstly, the data for G-Alexa without Trolox (filled circles) showed the saturation, and were consistent with that obtained at the single molecule level (SI Fig. 6). A similar saturation was also observed for cyt *c*-Alexa (triangles). In contrast, the corresponding data for G-Alexa obtained in the presence of Trolox (open circles) do not show the saturation until the excitation laser power of 1 W (photon flux of $\approx 0.6 \times 10^{27}$ $m^{-2} \cdot s^{-1}$). The lines in SI Fig. 7 were obtained by the fitting of the data to Eq. **1**. The k_{isc} and k_S values were fixed to 1.1×10^6 s^{-1} and 2×10^8 s^{-1}, respectively. The fitting gave k_T of $>10^9$ s^{-1} for G-Alexa in the presence of Trolox. In contrast, the fitting of the data for G-Alexa in the absence of Trolox, conducted by omitting a point at 1.5 W excitation, gave k_T of 1.2×10^5 s^{-1}. The significant acceleration of k_T in the presence of Trolox supported the pronounced quenching effect of T_1 by Trolox. We stress again that the k_T values should be considered as rough estimates mainly due to the uncertainty of the photon flux. However, the results clearly demonstrate that the relatively slow decay of T_1 is responsible for the saturation behavior observed for G-Alexa in the absence of Trolox. Interestingly, cyt *c*-Alexa also showed a saturation curve, whose fitting by assuming $k_{isc} = 1.1 \times 10^6$ s^{-1} and $k_S = 4 \times 10^8$ s^{-1} gave k_T of 6.7×10^5 s^{-1}. A larger k_S value compared to that of G-Alexa was used in the fitting, because S_1 is partly quenched by heme in cyt *c*-Alexa. The obtained k_T value demonstrates that heme in cyt *c*-Alexa cannot quench T_1 of Alexa efficiently.

We next investigated the effect of the triple state quencher on the fluorescence intensity fluctuation of G-Alexa. As reproduced in SI Fig. 8, the autocorrelation function of the single-molecule traces of G-Alexa observed in the absence of Trolox (filled circles) possessed a slow decay with a time constant of ≈75 ms. The corresponding single molecule traces of G-Alexa were obtained in the presence of 2 mM Trolox at the same excitation laser power (600 mW). The autocorrelation function for the data in the presence of Trolox (open circles) was systematically smaller than that obtained in the absence of Trolox, and the fitting of the data by a single exponential gave a time constant of ~11 ms. The results

demonstrate that the slow component of the autocorrelation function of G-Alexa in the absence of Trolox was partly caused by intensity fluctuations related to the appearance of T_1 in the photocycle.

Finally, we discuss that the effect of Trolox on the fluorescence intensity fluctuation of G-Alexa is distinct from that of heme in cyt *c*-Alexa. While the autocorrelation functions for G-Alexa in the presence of Trolox (SI Fig. 8) and for cyt *c*-Alexa (Fig. 5) show similar correlation times of ≈11 ms and ≈15 ms, respectively, they should be interpreted differently. In the case of G-Alexa in the presence Trolox, T_1 is selectively quenched as demonstrated in a significant increase in k_T ($>10^9$ s^{-1}) compared to that in the absence of Trolox (1.2×10^5 s^{-1}). In the case of cyt *c*-Alexa, the heme group primarily quenches S_1 of Alexa as demonstrated in the weakened fluorescence intensity. In contrast, the heme group does not quench T_1 of Alexa effectively as suggested in k_T (6.7×10^5 s^{-1}) that was similar to that of G-Alexa in the absence of Trolox. A further investigation is necessary for the detailed analysis of the autocorrelation functions; however, we suggest that the correlation function of cyt *c*-Alexa mainly reflects the quenching of S_1 by heme, and the dynamic changes in the Alexa-heme distance.

SI Materials and Methods. ***Bulk fluorescence measurements*.** The fluorescence intensities in the bulk conditions were observed by using the developed apparatus. The G-Alexa samples contained 83.4 pM G-Alexa, 20 mM Hepes at pH 7.5, 40 mM sodium chloride and 4 M Gdm with and without 2 mM Trolox (Calbiochem, Darmstadt, Germany). The cyt *c*-Alexa sample contained 83.4 pM cyt *c*-Alexa, 20 mM Hepes at pH 7.5, 40 mM sodium chloride and 1 M Gdm. All samples were degassed based on the procedure described in the main text. The laser power was monitored and adjusted by a laser power meter (Model 407A; Spectra Physics, Mountain View, CA). The time-dependent changes of the fluorescence images of the cell were detected on the EMCCD with a setting of the EM gain at 150. After the irradiation of laser, the images with an exposure time of 103 ms were consecutively recorded for ≈10 s. The average intensity of the flow pass at each time point was estimated. The fluorescence intensity at the moment immediately after the laser

excitation was obtained by the extrapolation of the average intensity to time 0 to minimize the effect of photobleaching.

Single-molecule fluorescence measurements. The fluorescence traces of G-Alexa in the presence of Trolox at the single molecule level were observed based on the method described in the main text. G-Alexa was prepared at a concentration of 48.2 fM in 20 mM Hepes at pH 7.5, 0.001% polyoxyethylene sorbitan monolaurate, 40 mM sodium chloride and 2 mM Trolox.

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