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Note

Förster Resonance Energy Transfer Between TMRbiocytin and Cy5-labeled Streptavidin

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Förster resonance energy transfer (FRET) is an energy transfer mechanism in which the energy of an excited fluorescent molecule (donor molecule) is transferred to another fluorescent material (acceptor molecule). This process takes place via dipole-dipole interaction, which is inversely proportional to the sixth power of the distance between the two dipoles. Therefore, FRET efficiency can be expressed as follows:[1,2]

$$E = \frac{1}{1+(r/R_0)^6} \quad (1)$$

where r is the distance between the energy donor and the acceptor, and R_0 (Förster distance) is the distance at which the energy transfer efficiency is 0.5. This distance depends on the energy donor and acceptor and is usually ~5 nm. The FRET efficiency can also be expressed as[3]

$$E = 1 - \frac{F'(D)}{F(D)} = 1 - \frac{\tau'(D)}{\tau(D)} \quad (2)$$

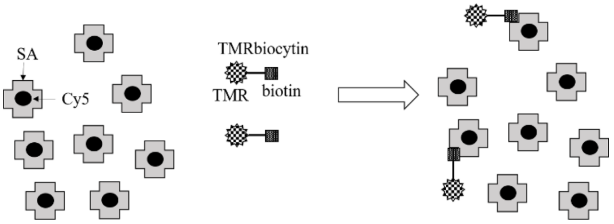
where F(D) and F'(D) are the fluorescence intensities in the absence and presence of a donor molecule, respectively, and τ(D) and τ'(D) are the fluorescence lifetimes in the absence and the presence of a donor molecule, respectively. Such energy transfer is sensitively dependent on distance in the range of 1 to 10 nm, which lies in the size range of most macromolecules (proteins, polymers, DNA, etc.). Therefore, FRET spectroscopy is a useful technique to obtain spatial information between specific sites in a macromolecule.[4,5] Consequently, FRET has many applications in the study of a wide variety of biophysical systems, including structural changes of proteins, protein-ligand binding, cell membrane studies, and chemosensory systems.[6,7]

The protein streptavidin (SA), widely used for various biotechnological applications, has a molecular weight of ~55 kDa and 4 binding pockets that can strongly bind to biotin.[8,9] Tetramethylrhodamine biocytin (TMRbiocytin) is a conjugate of biotin with a fluorescent dye and an 18-atom linker that is used to label streptavidin with a TMR dye.

In this study, we employed a time-resolved confocal microscope to investigate the FRET between TMRbiocytin and Cyanine 5 (Cy5) bound to SA. The average distance between the two fluorescent dyes was calculated using FRET efficiency, and the interaction between TMR and SA surface was discussed.

RESULTS AND DISCUSSION

As shown in *Scheme* 1, when an excessive amount of Cy5 labeled SA and TMRbiocytin are mixed, a single TMRbiocytin bound to SA is made. However, since each SA molecule has four biotin binding pockets, mixing with TMRbiocytin allows multiple TMRbiocytin molecules to bind to a single SA molecule. Owing to the binding of multiple TMRbiocytin molecules to one SA, self-quenching occurs between TMR molecules, which influences the fluorescence intensity of the donor molecule. Assuming that the binding of biotin to SA is noncooperative, the ratio of SA to multiple biotin molecules can be calculated statistically.[10] For example, when the mixing ratio of SA



***Scheme* 1.** Schematic showing the binding of TMRbiocytin to Cy5 labeled SA.

to biotin is 1:1, the ratio of SA with one biotin to SA with multiple biotin molecules is 0.59. Further, increasing the mixing ratio of SA increases the number of SAs with multiple biotins. However, increasing the mixing ratio of SA also increases the number of Cy5 dye molecules bound to SA, which also affect the fluorescence of donor molecules even though the fluorescence of Cy5 is mostly blocked by the interference filter. Therefore, the ratio of TMRbiocytin to Cy5_SA must be carefully determined. In our experiment, the ratio of Cy5_SA to TMRbiocytin was determined to be 5:1, whereby the influence of self-quenching and unwanted fluorescence of Cy5 on the FRET analysis was minimized. Notably, the biotinylated dye fluorescence is quenched in the presence of SA and recovers when empty binding pockets are occupied by biotins.[11] Therefore, to measure the fluorescence change of TMRbiocytin arising from FRET, we removed the fluorescence quenching of TMRbiocytin by SA by adding an excess of biotin to the TMRbiocytin-Cy5_SA complex.

Fluorescence correlation spectroscopy (FCS) experiments were performed to confirm the binding of TMRbiocytin to Cy5_SA. The fluorescence correlation spectrum is obtained by autocorrelation of fluorescence fluctuations occurring in a very small volume (~1 fL) caused by the focus of the objective lens.[12] These fluctuations in fluorescence intensity are typically caused by physical and chemical properties of the sample, including diffusion, concentration changes, and chemical reaction rates of the sample. The autocorrelation function for fluorescence intensity is defined as the product of the average value of fluorescence intensity between time t and t+τ as shown in the following equation.

$$G(\tau) = \frac{\langle F(t)\rangle\langle F(t+\tau)\rangle}{\langle F\rangle^2} = 1 + \frac{\langle \delta F(0)\delta F(\tau)\rangle}{\langle F\rangle^2} \quad (3)$$

where δF(t) = <F>-F(t) is the fluctuation in fluorescence intensity at time *t*. If the laser beam is a Gaussian ellipsoid with radius *s* and height *u*, autocorrelation caused by free diffusion in 3D is given by:

$$G(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\left(\frac{s}{u}\right)^2\frac{\tau}{\tau_D}\right)^{-\frac{1}{2}} = \frac{1}{N}D(\tau) \quad (4)$$

where *N* is the average number of fluorescent molecules in the laser focus, and τ_D is the diffusion time given by the following equation.

$$\tau_D = \frac{s^2}{4D} \quad (5)$$

where *D* is the diffusion coefficient and is calculated using the Stokes–Einstein equation:

$$D = \frac{kT}{6\pi\eta r} \quad (6)$$

where κ is the Boltzmann constant, *T* is the temperature, η is the viscosity coefficient, and *r* is the hydrodynamic radius of the diffusing species.

Fig. 1 shows the normalized FCS curves of TMR, TMRbiocytin, and TMRbiocytin-Cy5-labeled SA. As the molecular weight of the sample increases, the autocorrelation curve shifts to the right according to equation (4). *Table* 1 shows the parameters of equation (4) that fit the FCS curves shown in *Fig.* 1. The diffusion times of TMR, TMRbiocytin, and TMRbiocytin-Cy5_SA were determined to be 71 μs, 92 μs, and 354 μs, respectively. The diffusion of TMRbiocytin was slower than that of TMR because the lysine linker and biotin were bound to TMR, and the diffusion of TMRbiocytin was much slower with TMRbiocytin bound to Cy5_SA. As the radius of the laser focus was constant during the experiment, equation (4) could be written as $\sqrt{D_1\tau_1} = \sqrt{D_2\tau_2}$, and the diffusion coefficients of TMRbiocytin and TMRbiocytin-Cy5_SA were calculated using the known diffusion coefficient of TMR (2.6×10^{-10} m^2/s at 25 °C).[13] Using equation (6), the diffusion coefficients of TMRbiocytin and TMRbiocytin-Cy5_SA were determined to be 2.00×10^{-10} m^2/s and 0.52×10^{-10}

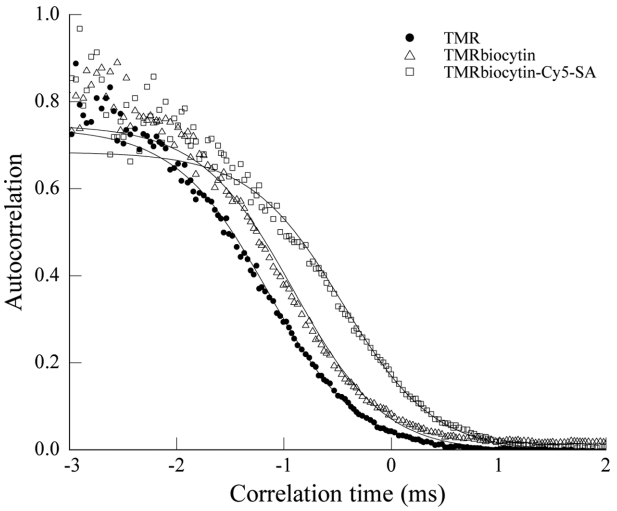


***Figure* 1.** FCS curves of TMR (solid circle), TMRbiocytin (open triangle), TMRbiocytin-Cy5_SA (open rectangle) along with the fitting curves using equation (4).

***Table* 1.** Fitting parameters of FCS curves shown in *Fig.* 1

	N	τ_D (μs)
TMR	12.0±0.1	71.2±1.1
TMRbiocytin	17±1	91.8±7.6
TMRbiocytin-Cy5_SA	23.3±0.38	354±11

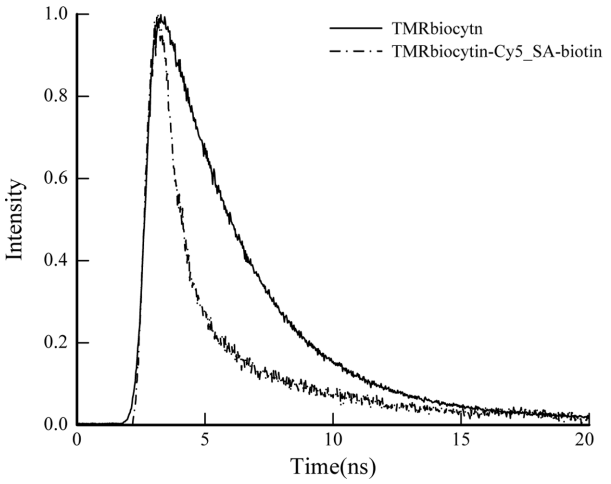


***Figure* 2.** Fluorescence decay of TMRbiocytin and TMRbiocytin-Cy5_SA in the presence of excess biotin.

m^2/s, respectively, and the hydrodynamic radius of TMRbiocytin-Cy5_SA was calculated to be 4.63 nm. These results confirmed that TMRbiocytin was bound to Cy5_SA.

Fig. 2 shows the fluorescence decay of TMRbiocytin and TMRbiocytin-Cy5_SA in the presence of excess biotin. The time-resolved fluorescence decay can be fitted as a sum of single exponential decays given by:[14]

$$I(t) = \sum_{i=1}^{n} a_i \exp\left(-\frac{t}{\tau_i}\right) \quad (7)$$

where a_i is the amplitude of the ith component at t=0, ($\sum_{i=1}^{n} a_i = 1$) and the average fluorescence lifetime (τ_a) is $\sum_{i=1}^{n} a_i \tau_i$.

The fluorescence decay of TMRbiocytin fitted a single exponential function with the fluorescence lifetime of 3.41 ns. When Cy5_SA was bound to TMRbiocytin, its fluorescence decay data fitted a double exponential function with an average fluorescence lifetime of 1.66 ns. The fluorescence quenching of TMRbiocytin was attributed to the FRET process between TMR and Cy5 as the addition of excess biotin eliminated fluorescence quenching by SA in this sample. Based on equations (1) and (2), and the Förster distance between TMR and Cy5 (R_0 = 5.3 nm), FRET efficiency was estimated to be 1-(1.66 ns/3.41 ns) = 0.51, and the distance between TMR and Cy5 bound to SA was calculated to be 5.4 nm.[15] TMRbiocytin is considered to be relatively flexible because it has a single bond carbon chain of ~2 nm length between TMR and biotin. As the hydrodynamic radius of SA measured using FCS was 4.65 nm, it was evident that the TMR portion of TMRbiocytin was not attached to the SA surface. Therefore, there was little interaction between the SA surface and TMR when the binding pockets of TMRbiocytin-Cy5_SA complex were all occupied by excess biotin. As Cy5 binds to one NH_2 group among several lysine residues of SA, the site to which Cy5 is bound may differ for each SA, which implies that the distance between the two fluorescent molecules determined by FRET is an average value. In conclusion, this study showed that a significant amount of FRET occurred between TMRbicytin and Cy5_SA, and the distance between the two fluorescent dyes was measured to be ~5.4 nm. This distance indicated that there was no appreciable interaction between TMR and Cy5 bound to SA. As this study investigated FRET in a bulk solution, the distribution of FRET efficiency of individual molecules and the change in fluorescence occurring in real time could not be measured. Using single molecule spectroscopy, we can better understand the dynamic changes of individual TMRbiocytin-Cy5_SA, which is the focus of our future research.

EXPERIMENTAL

SA used in this experiment was purchased from Agilent (USA) and dissolved in 10 mM $NaHCO_3$. Cy5-labeled SA was obtained by mixing Cy5-*N*-hydroxysuccinimide (Cy5-NHS, GE Healthcare) dissolved in dimethylformamide (Sigma Aldrich) and SA in a molar ratio of 3:1 for 30 min, and subsequently removing unreacted Cy5-NHS from the sample using a size exclusion column (Centri-spin 20, Princeton separation). The degree of labeling determined by UV spectroscopy was ~1.2 Cy5 per SA. Fluorescence experiments were performed with a time-resolved confocal fluorescence microscope that consisted of an inverted fluorescence microscope (Olympus IX71) and a 63× alpha Plan-Apochromat total internal reflection fluorescence (TIRF) objective (Zeiss). The details of the microscope setup are described elsewhere.[16] In brief, the sample was excited

***Table* 2.** Fitting parameters for fluorescence decays of TMRbiocytin and TMRbiocytin-Cy5_SA in the presence of excess biotin using equation (7)

	a_1	a_2	τ_1 / ns	τ_2 / ns	τ_a / ns
TMRbiocytin	-	-	3.41	-	3.41
TMRbiocytin-Cy5_SA (excess biotin)	0.50	1.50	4.30	0.77	1.66

by a pulsed diode laser (510 nm, Picoquant) with a pulse duration time of ~100 ps. The sample was then placed on a glass coverslip, and a supported lipid bilayer (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, POPC, Avanti Polar Lipids) was coated on the surface to prevent the adsorption of Cy5-labeled SA and TMRbiocytin on the glass surface. The fluorescence generated from the sample was collected by the same objective lens, passed through several lenses and a pinhole (75 μm, Thorlabs), and reached a GaAs photomultiplier tube (Hamamatsu, H7421-50). The photon arrival time was recorded by a single photon counting board (Picoquant, Timeharp 260 PICO) to obtain fluorescence correlation and time-resolved fluorescence. Data collection and analysis was carried out using SymPhoTime 64 software (Picoquant).

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