Simultaneous dual-color and dual-polarization imaging of single molecules

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(Received 9 August 2000; accepted for publication 16 October 2000)

We report the observation of single-molecule colocalization and quantitative fluorescence resonant energy transfer by simultaneously imaging the emission and polarization characteristics of two colocalized fluorophores using a simple optical design. The methodology was tested using the ligand-receptor system streptavidin, fluorescence labeled with the dye Cy5, and biotin labeled with tetramethylrhodamine. Discrimination of the two dyes permitted the observation of single-pair fluorescence resonant energy transfer with an efficiency of 89%. The multidimensional character of our fluorescence microscopy combined with the robustness of our design provides a simple method suitable to study biomolecular interactions on the single molecule level. © *2000 American Institute of Physics.* [S0003-6951(00)01451-0]

Fluorescence microscopy has many distinct advantages for the study of biological interactions. In particular, when the detection sensitivity is increased to the single-molecule level, it can retrieve properties averaged out in ensemble measurements.[1] A potentially important extension of single molecule imaging is utilization of a multicolor approach which permits simultaneous observation of interacting partners. Such colocalization studies with specific labeling of molecules that emit different colors have been reported previously.[2,3] Colocalization has also been studied at the nanometer scale by single-pair fluorescence resonant energy transfer (spFRET).[2–4] SpFRET has been demonstrated to measure intramolecular distances,[5,6] to observe conformational molecular dynamics,[2,6,7] and to determine local ion concentrations.[8,9] However, for both colocalization and spFRET studies, simultaneous observation of the relative orientation of the fluorophores would permit more detailed access to the molecular interactions involved. For example, a correlation found in the rotational dynamics of colocalized molecules would give insight about their aggregation, size, and organization into larger complexes. Further, orientational information would also render distance measurements by spFRET that are model independent[5,6] and thus more accurate. Hence, there is an immediate need for a simple and robust ''multidimensional'' single-molecule imaging dedicated to biosciences.

Here we show a compact design for a dual-color and dual-polarization experiment for single molecule microscopy. Compared to the dual-view microscopy concept by Kinosita *et al.*,[10] our approach combines polarization and color discrimination using a very simple design, and compared to commercial multicolor cameras, it uses a highly sensitive and low noise charge coupled device (CCD) camera for single-molecule imaging. The design is suitable for in-plane rotational diffusion studies of colocalized molecules,[11–13] and for the determination of the in-plane orientations of the donor and the acceptor during spFRET.

For demonstration of the applicability of our concept in a biological system, we used the ligand-receptor pair formed by biotin and streptavidin.[14] This pair is frequently used in antibody based biological assays and for *in situ* hybridization assays. Streptavidin is a protein that has four binding sites for biotin. A 3 nM solution of Cy5-labeled streptavidin (Amersham Pharmacia Biotech) was deposited onto a glass slide. The charged surface of the streptavidin resulted in an unspecific immobilization of the protein onto the glass substrate. Subsequently, the sample was incubated with a 1 nM solution of tetramethylrhodamine (TMR)-labeled biotin (Molecular Probes).

The experimental setup was derived from that described previously.[11,15] In short, the samples were mounted onto an inverted microscope (Zeiss) equipped with a 100× oil-immersion objective (NA=1.4, Zeiss), and alternatingly illuminated for 10 ms with a light of 514 nm wavelength (Ar^+ laser, Spectra Physics) for excitation of TMR and of 640 nm (dye laser, Spectra Physics) for direct excitation of Cy5. The illumination intensity was set to 4 kW/cm^2 at 514 nm and 2 kW/cm^2 at 640 nm. The polarization of the excitation light was adjusted by a Berek Polarizer (New Focus). Use of an appropriate filter combination (customized dual colors band pass filters, Chroma Technology) permitted the fluorescence images to be clearly distinguished from scattered light. The color and polarization of the light emitted were discriminated by a combination of a custom-made dichroic wedge mirror (3° angle, center wavelength of 630 nm, Chroma Technology) and a Wollaston prism (WO-1.5°-NS-10, Zeta Internal., 1.5° separation), both placed in the infinity path of the microscope (see Fig. 1). A 10 cm achromatic lens (PAC073, Newport) projected the images onto a nitrogen-cooled back-illuminated CCD camera (LN/CCD-400-PB, Princeton Instruments), simultaneously forming four images (see Fig. 2): the donor fluorescence (TMR in the green channel) and the acceptor fluorescence (Cy5 in the red channel) both being split into two polarization images. Using 10 ms illumination time per acquisition, those four images (4×60×60 pixels) were recorded at a rate of 6.7 Hz in a continuous mode which could be increased to 85 Hz by image shifting on the CCD. Crosstalk between the two color channels was negli-

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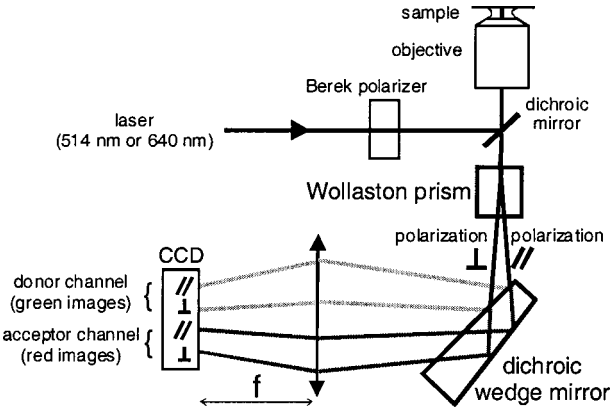

FIG. 1. Experimental setup. Four images are simultaneously formed on the CCD camera by use of a Wollaston prism and a dichroic wedge mirror in the infinity beam path.

gible compared to the detection efficiency[16] $\eta_{\mathrm{TMR}}=0.10$ for TMR in the green channel and $\eta_{\mathrm{Cy5}}=0.073$ for Cy5 in the red channel ($\eta_{\mathrm{TMR}}<0.003$ for TMR in the red channel, and $\eta_{\mathrm{Cy5}}<0.004$ for Cy5 in the green channel). The low value of this crosstalk was confirmed by using samples with only one of the two fluorophores. It assured that no TMR molecules were detectable in the Cy5 channel and vice versa (see also Fig. 2). By measuring the reflection spectra of both the dichroic wedge mirror and the dichroic mirror for both polarizations, the ratio of the detection efficiencies η in the parallel ($\|$) and perpendicular ($\perp$) channels was determined to be $g_{\mathrm{TMR}}=\eta_{\perp}/\eta_{\|}=0.87$ in the green channel and $g_{\mathrm{Cy5}}=0.96$ in the red channel. An analysis program determined the position of each signal in the four images by fitting to a two-dimensional Gaussian surface.[15] The photoncounts F were determined with a precision of $\sim$20%.

The dual-color dual-polarization detection yielded three types of information: (i) the properties of the fluorescence-labeled ligand (biotin) in the green channel using 514 nm illumination, (ii) the properties of the fluorescence-labeled protein (streptavidin) in the red channel using 640 nm illumination, and (iii) on 514 nm illumination, the dynamics of energy transfer between the two fluorophores.

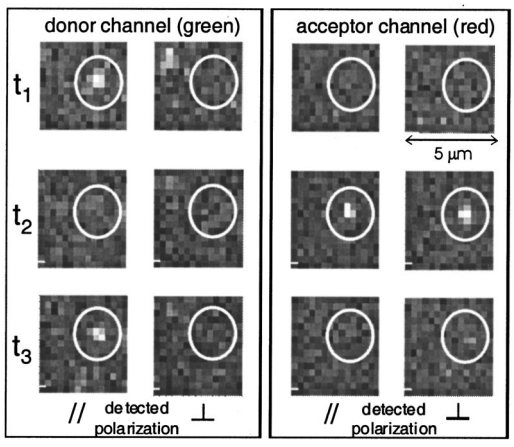

FIG. 2. Consecutive sequence of three dual-color and dual-polarization images of TMR biotin (donor) and Cy5 streptavidin (acceptor) showing spFRET. All images were taken with an illumination of 514 nm for 10 ms with 140 ms delay. The acceptor (visible at t_2) is oriented 45° with respect to the donor (visible at t_1 and t_3). Image intensity scales: 0 (black)–100 (white) counts in the red channel and 0–150 counts in the green channel.

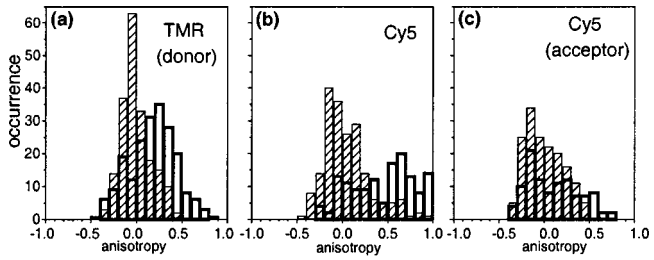

FIG. 3. Histograms of polarization anisotropy of (a) TMR on 514 nm excitation, (b) Cy5 on 640 nm excitation and (c) Cy5 on spFRET. Hollow bars correspond to linear polarized excitation and shaded bars to circular polarized excitation.

Individual TMR-biotin molecules were analyzed in the green images with either linear or circular polarized illumination light at 514 nm. The discrete peaks of a width corresponding to the point-spread function of the microscope are assigned to individual TMR-biotin molecules characterized by an average signal of 41 ± 8 counts/ms[15] and by single step photobleaching events. In Figs. 3(a) and 3(b), the distributions of the polarization anisotropies defined by $r=(F_{\|}-F_{\perp})/(F_{\|}+2F_{\perp})$ are shown for TMR. On average, the mean polarization anisotropy of TMR biotin is $\langle r_{\mathrm{TMR,lin}}\rangle=0.18\pm0.03$ on linear polarized excitation, and on circular polarized excitation $\langle r_{\mathrm{TMR,circ}}\rangle=0.00\pm0.02$. Analogous analysis was performed for signals of Cy5 streptavidin using 640 nm illumination. The count rate is characterized by 30 ± 5 counts/ms. On linear excitation the mean polarization anisotropy is $\langle r_{\mathrm{Cy5,lin}}\rangle=0.48\pm0.10$ and on circular polarized excitation $\langle r_{\mathrm{Cy5,circ}}\rangle=0.06\pm0.03$.

The high anisotropy of $\langle r_{\mathrm{Cy5,lin}}\rangle$ indicates that the Cy5 label of the streptavidin is immobile. This finding was confirmed by successive imaging on a time scale of up to $\sim$100 ms, with linear and circular polarized excitation: fixed and well-defined dipoles were observed (data not shown). TMR, the label on the ligand, is found to be rotationally mobile. From both the polarization anisotropy ($\langle r_{\mathrm{TMR,lin}}\rangle=0.18$) and the fluorescence lifetime ($\tau_f=2.1$ ns),[15] and assuming that in the rest frame of the fluorophores the emission and absorption dipoles are parallel, the TMR rotational mobility is characterized by a rotational diffusion constant of $\sim10^8$ rad^2/s.

An example for a single-pair FRET is shown in Fig. 2. Three sets of consecutive images (at t_1, t_2, and t_3) are obtained using linear illumination at 514 nm. At t_1, in the donor emission channel (left), the fluorescence of a single TMR-biotin molecule is present which is oriented along the illumination polarization. No fluorescence is identified in the red images (right). At t_2, spFRET occurs: the fluorescence from the TMR-biotin molecule is quenched, and at the corresponding position in the red channel, fluorescence from a Cy5-streptavidin molecule appears. It should be noted that the polarization of the immobile acceptor Cy5-streptavidin is 45° with respect to that of the TMR biotin. At t_3, energy transfer has stopped, and the TMR fluorescence is recovered to the level as at t_1.

Figure 4 is a summary of a similar experiment. The fluorescence of a Förster pair is shown as a function of time when alternating the color of illumination between red (640 nm) and green (514 nm). Occurrence of spFRET is confirmed by the anticorrelation between TMR and Cy5. Ne-

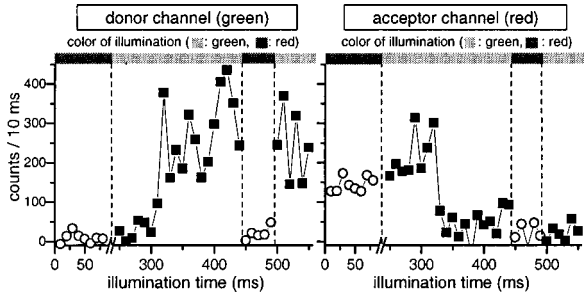

FIG. 4. Fluorescence emitted by a spFRET pair and detected in the donor (left) and acceptor (right) channels as a function of time. The excitation was alternated between 514 nm (empty circles) and 640 nm (filled squares). For both wavelengths, each point corresponds to 10 ms circular polarized excitation. Anticorrelation between the TMR molecule (donor) and the Cy5 molecule (acceptor) shows the presence of spFRET. After 330 ms illumination, the Cy5 molecule bleached and spFRET stopped.

glecting crosstalk between the two channels, the spFRET efficiency E is given by[6,9]

$$E=\left(1+\frac{\eta_{\mathrm{Cy5}}}{\eta_{\mathrm{TMR}}}\frac{\phi_{\mathrm{Cy5}}}{\phi_{\mathrm{TMR}}}\frac{F_{\mathrm{TMR}}}{F_{\mathrm{Cy5}}}\right)^{-1},$$

where the quantum yield of the two fluorophores, ϕ_{Cy5} and ϕ_{TMR} are both equal to 0.28 (Amersham Pharmacia Biotech and Ref. 15). For the pair shown in Fig. 4, the mean efficiency is thus $\langle E\rangle=0.89\pm0.08$.

Polarization anisotropy of the spFRET was further analyzed. Figure 3(c) shows the distribution of the polarization anisotropy of the acceptor Cy5 streptavidin when spFRET occurs, i.e., on green illumination. The mean polarization anisotropy is, on linear excitation, $\langle r_{\mathrm{FRET,lin}}\rangle=0.09\pm0.04$. When compared to the corresponding value for the donor fluorescence, $\langle r_{\mathrm{TMR,lin}}\rangle=0.18$, the mean anisotropy is reduced by a factor of two, a well known additional depolarization of FRET.[4] On circular excitation, $\langle r_{\mathrm{FRET,circ}}\rangle=0.00\pm0.03$, as for the donor only.

One objective of spFRET is to measure the distance between the donor and the acceptor d by means of the spFRET efficiency[17]

$$d^6=8.79\times10^{23}(n^{-4}\cdot\phi_{\mathrm{TMR}}\cdot J\cdot\kappa^2)\left(\frac{1}{E}-1\right)\quad(\text{in Å}^6),$$

where J is the spectral overlap of the donor emission and the acceptor absorption (measured in M^{-1} cm^3), n is the refractive index of the medium, and κ is an orientation factor which accounts for the relative orientation of the donor and acceptor dipole. The relative orientation of the projection of the donor and the acceptor dipoles onto the plane perpendicular to the direction of the excitation light is determined *in situ* by our system for each individual Förster pair (and in general for two colocalized molecules). Hence, our setup permits us to perform the direct test on each spFRET pair, whether one or both fluorophores are freely rotating on a time scale comparable or faster than the fluorescence lifetime. For freely rotating pairs, $\kappa^2=2/3$, a value frequently assumed in FRET studies.[2,5,8,17] As an example, the spFRET pair shown in Fig. 2 has a factor κ^2 different from 2/3 given that the donor emission is polarized and the acceptor is immobile. In this case, κ^2 could only be evaluated if the relative orientations are determined with respect to a second projection plane or by introduction of an assumption for the molecule's orientation itself. The latter might be obtained, with circular polarized excitation from the single molecule fluorescence intensity which scales with the out-of-plane angle α like $\cos^2(\alpha)$.

In conclusion, the work presented in this letter demonstrates a simple and robust optical setup to simultaneously observe various parameters of colocalized molecules: their position, color, fluorescence signal, and in-plane orientation at an image rate of 6.7 Hz (and up to 85 Hz). Any pair of fluorophores could be imaged similarly using the appropriate coatings on the dichroic wedge. The imaging frequency is basically limited by the signal-to-noise ratio for a given illumination time (in practice $\sim$1 kHz). The imaged field is limited by the magnification and the size of the CCD. In our case its size is $\sim20\times20$ $\mu\mathrm{m}^2$, perfectly matching the needs of cell-based assays. Given that versatility, the setup provides an effective method to study molecular interactions on the single molecule level in a biological environment (e.g., proteins in cell membranes).

This work was supported by funds from the Dutch FOM/ALW/NWO program for Physical Biology (T.S.). One author (L.C.) acknowledges support from the European Marie-Curie fellowship program.

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