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Two-photon Excitation Induced Fluorescence of a Trifluorophore-labeled DNA¶

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ABSTRACT

Two-photon excitation of a trifluorophore (6-carboxyfluorescein, *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine and cyanine-5 monofunctional dye) labeled DNA, which has a scaffold of 26 nucleotides, was achieved using focused laser light of a Q-switched Nd-YAG laser (1064 nm). The observed fluorescence signature (emission ratio from the three fluorophores) of the labeled DNA after two-photon excitation is very different from the fluorescence signatures produced by one-photon excitation at different wavelength. The additional fluorescence signatures produced by two-photon excitation of the fluorescent oligonucleotides will facilitate their use as combinatorial fluorescence energy transfer tags for multiplex genetic analysis.

INTRODUCTION

The need to study many biological targets simultaneously has driven the development of multiplex fluorescence tags (1). However, the number of available fluorescence dyes that have distinguishable emission spectra is limited, and therefore, researchers have developed combinatorial fluorescence energy transfer tags (CFET) by exploiting energy transfer and combinatorial synthesis (2–4). Fluorescence energy transfer has been used extensively as a spectroscopic ruler for biological structures (5–7), and energy transfer primers and terminators are markedly superior to single dye–labeled reagents in DNA sequencing and analysis (8,9). Recently, we developed a novel approach for constructing a large number of CFET from a small number of chromophores for multiplex biological assays (2,3,10). We showed that with three fluorophores [6-carboxyfluorescein (**F**), *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine (**R**) and cyanine-5 monofunctional dye (**Cy**)] using a single excitation wavelength (488 nm; Ar-ion laser), eight CFET tags with unique fluorescence signatures can be constructed and detected by a three-color capillary array electrophoresis system (3). The number of distinguishable fluorescence signatures can be increased using additional excitation wavelengths, such as 543 nm (He–Ne laser) and 633 nm (He–Ne laser), thereby adding more parameters to distinguish the targets.

In this report, we present a novel approach to further increase the number of distinguishable parameters of CFET tags using multiphoton absorption techniques. Traditionally, multiplex fluorescence tags are excited at a wavelength where the chromophores have a strong absorption, *e.g.* 488 nm for fluorescein derivatives. Excitation usually involves the absorption of only one photon at a time. However, with intense sources of irradiation, such as pulsed lasers, the probability of simultaneous absorption of two (or more) photons becomes finite (11). In principle, one can use the combined energy of two lower energy photons of ''long wavelength'' to produce excited states of the fluorescence tags, which normally absorb only one higher energy photon of ''short wavelength'' (Scheme 1). For example, two near IR photons of the first harmonic of a YAG laser (λ = 1064 nm, 26.9 kcal/mol) possess the same energy as a single green photon (λ = 532 nm, 53.8 kcal/mol). Two- (and multi-) photon absorption is gaining interest in a number of interdisciplinary areas, such as fluorescence imaging, phototherapy and biophotonics (12,13), because of its many advantages over traditional one-photon absorption. For two-photon absorption, IR light can be used, which allows deep penetration into biological targets such as tissue and organs. In addition, in fluorescence detection methods, a low background is achieved because of large separation of excitation and fluorescence emission signals, which also allows the use of wideband filters for fluorescence detection. Because two-photon absorption shows a quadratic dependence on the excitation light, focused light beams are often used, which makes spatial isolation of the excitation event possible. This spatial resolution can be used for three-dimensional fluorescence imaging. In addition, damage and photobleaching due to excitation is confined to the small submicron volume in which two-photon excitation occurs. In this study, we report the two-photon excitation of a trifluorophore-labeled DNA, which has a scaffold of 26 nucleotides, using the focused laser light of a Q-switched Nd-YAG laser (1064 nm) that produces additional fluorescence signatures to distinguish the labeled DNA targets.

MATERIALS AND METHODS

The trichromophore-labeled DNA (**F-3–R-7–Cy-13**) that has a scaffold of 26 nucleotides (Scheme 2) was constructed by solid-phase phosphoramidite chemistry on a DNA synthesizer and solution coupling chemistry. **F** and **R**

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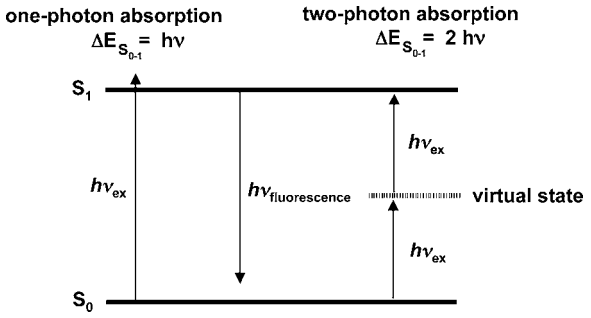
Abbreviations: CFET, combinatorial fluorescence energy transfer tags; CW, continuous wave; dT, deoxythymidine; NHS, *N*-hydroxy succinimidyl; TBE, buffer containing 0.89 M tris(hydroxymethoxy)aminomethane, 0.89 M boric acid and 0.02 M ethylenediaminetetraacetic acid, pH = 8.0.



Scheme 1. State energy diagram, where S_0 is the ground state and S_1 the first excited singlet state.

were incorporated into the oligonucleotides with Fluorescein-deoxythymidine and *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine-deoxythymidine (TAMRA-dT) phosphoramidites (Glen Research, Sterling, VA). A modified thymidine with an amino linker (amino-modifier C_6dT; Glen Research) was incorporated into the oligonucleotide for coupling with Cy5 *N*-hydroxy succinimidyl (NHS) ester (Cy5 monofunctional dye; Amersham Bioscience, Piscataway, NJ). Unmodified dT was used as spacer between the fluorophores. To incorporate Cy5 into the CFET tag, 5–8 nmol of **F**- and **R**-labeled oligonucleotides containing an amino linker in 33 μL of 0.25 *M* Na_2CO_3–$NaHCO_3$ buffer, pH 9.0, were incubated for 3 h at room temperature with a ~45-fold excess of Cy5 NHS ester in 12 μL anhydrous dimethyl sulfoxide. Unreacted dye was removed by size exclusion chromatography on a PD-10 column (Amersham Bioscience). **F-3–R-7–Cy-13** was purified by gel electrophoresis and desalted with an oligonucleotide purification cartridge. The structure of **F-3–R-7–Cy-13** was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry.

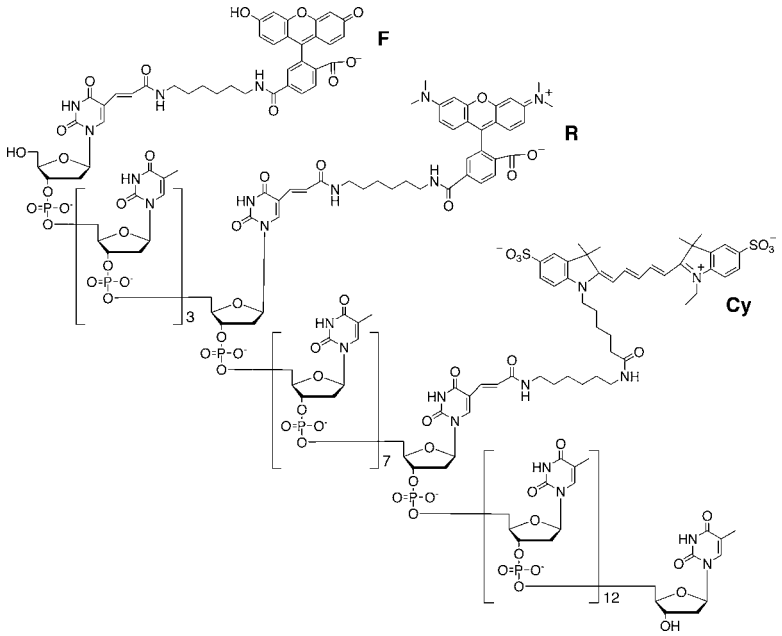
The fluorescence spectra were recorded as follows. The sample solution (3 mL, 0.3 μ*M*) was placed in a 2 × 1 cm fluorescence cell and stirred continuously. For two-photon excitation experiments, the pulses of a Spectra Physics (Mountain View, CA) GCR-150-30 Nd-YAG laser (1064 nm, *ca* 30 mJ/pulse, 10 ns) were focused in the center of the sample cell. The emitted light was collected with a series of lenses and focused on a fiber optic, which couples the fluorescence light into the CCD detector system (USB2000; Ocean Optics Inc., Dunedin, FL). For one-photon excitation experiments, a green diode laser emitting at 532 nm (green laser pointer) or a 150 W Halogen lamp in conjunction with a monochromator (Jarrell Ash, Grand Junction, CO) set at 488 nm were used.

RESULTS AND DISCUSSIONS

Figure 1a shows the optical absorption spectrum of the trichromophore-labeled DNA, **F-3–R-7–Cy-13** (Scheme 2). The spectrum is composed of the three chromophores **F** (λ_{abs}^{max} = 496 nm), **R** (λ_{abs}^{max} = 555 nm) and **Cy** (λ_{abs}^{max} = 643 nm), which is in agreement with the absorption spectra of the individual dyes (Fig. 1b). In our previous study, we used 488 nm as excitation wavelength for the dye-labeled DNA (2), where **F** possesses a strong absorption. Figure 2a shows the fluorescence spectrum of **F-3–R-7–Cy-13** after excitation at 488 nm, which exhibits three fluorescence peaks corresponding to fluorescence of **F** (λ_{em}^{max} = 525 nm), **R** (λ_{em}^{max} = 585 nm) and **Cy** (λ_{em}^{max} = 670 nm). We have shown that this unique fluorescence signature is generated by triple fluorescence energy transfer, after excitation of the fluorescein chromophore (2).

If **F-3–R-7–Cy-13** is excited at 1064 nm using an unfocused pulsed laser beam (30 mJ/pulse; beam diameter 7 mm), where **F-3–R-7–Cy-13** does not absorb (Fig. 1a), as expected, no fluorescence was observed. But if the laser beam is focused inside the sample solution using identical laser intensity (same pulse power), an appreciable amount of fluorescence was observed (Fig. 2c). This is a strong evidence for multi-photon excitation. The fluorescence spectrum after excitation with focused laser light at 1064 nm (Fig. 2c) shows a completely different signature compared with the fluorescence spectrum at 488 nm excitation (Fig. 1a). After multi-photon absorption (1064 nm) essentially no fluorescence of **F** was observed (Fig. 2c), whereas the fluorescence of **F** is strongest for 488 nm excitation (Fig. 2a).

Because two photons of 1064 nm possess the same energy as one photon at 532 nm, the solutions of **F-3–R-7–Cy-13** were excited at 532 nm using a continuous wave (CW) laser diode. The observed spectrum (Fig. 2b) shows no fluorescence of **F** (similar to the spectrum observed after 1064 nm excitation) (Fig. 2c). However, the ratio of the fluorescence intensities of **R** and **Cy** are different for 532 and 1064 nm excitation, which is caused by



Scheme 2. Molecular Structure of the trifluorophore-labeled DNA **F-3–R-7–Cy-13**.

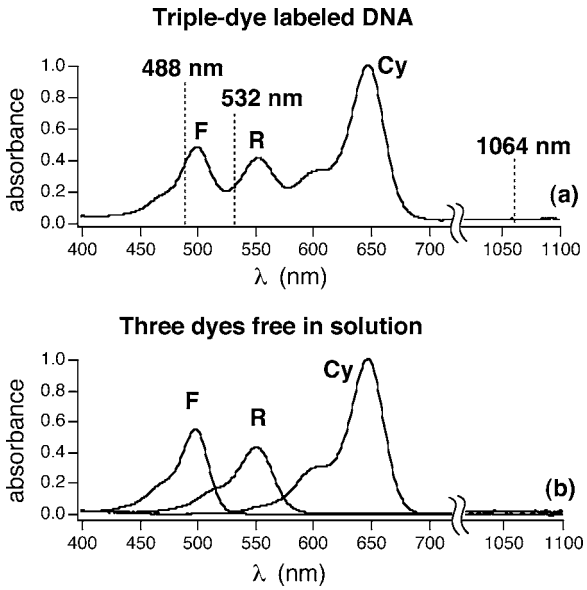


Figure 1. Optical absorption spectra of **F-3–R-7–Cy-13** (a) and an equimolar solution of a mixture of **F**, **R** and **Cy** (b).

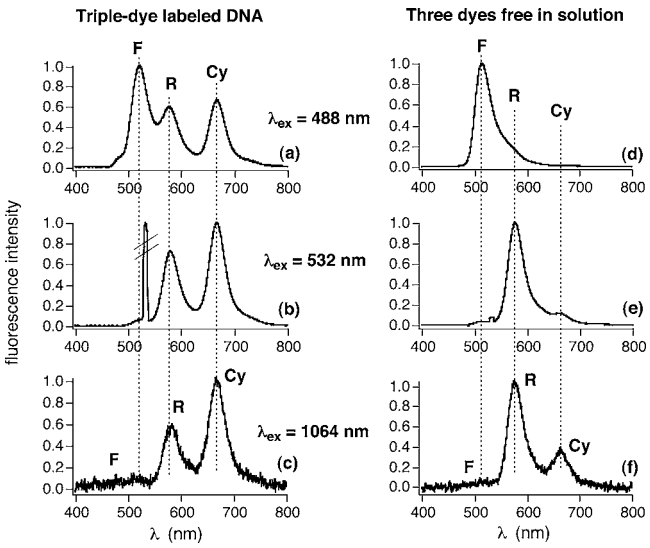


Figure 2. Fluorescence spectra after excitation of **F-3–R-7–Cy-13** (a,b,c) and an equivalent mixture of **F**, **R**, **Cy** (d,e,f) in aqueous buffer solution (1 × TBE buffer, pH = 8.5) at 488 nm (a,d), 532 nm (b,e) and focused laser pulses at 1064 nm (c,f). The spike at 532 nm in Spectrum b is caused by the scattering of laser light into the detector.

different ratios for one-photon and two-photon absorption efficiency of **R** and **Cy** (see below).

Control experiments were performed in which mixtures of equimolar concentrations of the three dyes, **F**, **R** and **Cy**, free in solution were excited with CW light of 488 nm, 532 nm and focused pulsed laser beams of 1064 nm (Fig. 2d–f). After 488 nm excitation, only **F** shows fluorescence (Spectrum d). The trace fluorescence of **R** is caused by residual absorption of **R** at 488 nm (Fig. 1b). No fluorescence was observed for **Cy**, indicating that no fluorescence energy transfer occurs. The lack of energy transfer is expected because of the low concentration of the dyes (μM) and short fluorescence lifetime of **F** ($\tau_f \sim 4.9$ ns) (14). Excitation at 532 nm shows almost exclusive emission of **R** together with small emission of **Cy** (Fig. 2e), which is caused by the small absorption of **Cy** at 532 nm. Irradiation of the three-dye mixture with focused laser beams at 1064 nm shows strong fluorescence of **R** together with an appreciable amount of fluorescence of **Cy** (Fig. 2f). No fluorescence of **F** was observed, which is in agreement with the fluorescence signature of the triple dye–labeled DNA (Fig. 2c). The two-photon cross section for fluorescein at 1064 nm is known to be low ($\delta = 0.075 \times 10^{-50}$ cm^4 s/photon) (15–17), whereas for rhodamine B, it is orders of magnitudes higher ($\delta = 14 \times 10^{-50}$ cm^4 s/photon) (15,17,18). Therefore, using focused laser beams at 1064 nm, efficient excitation was achieved for **R** but not for **F** in **F-3–R-7–Cy-13** and the mixture of the free dyes (Fig. 2c,f).

An appreciable amount of fluorescence of **Cy** was observed after excitation of the mixture of the three dyes free in solution at 1064 nm, whereas excitation of the same solution at 532 nm showed only little fluorescence of **Cy**. Comparison of the fluorescence intensity of **Cy** with **R** after excitation at 1064 nm suggests that **Cy** possesses a two photon–cross section in the order of $\delta \sim 10^{-50}$ cm^4 s/photon at 1064 nm, which is significantly higher than for **F**, but lower than for **R** at 1064 nm. Figure 3 demonstrates that the fluorescence of the three-dye mixture after excitation with focused laser pulses at 1064 nm (Figs. 2f and 3a) is composed of the fluorescence of the two individual dyes, **R** and **Cy** (Fig. 3b,c), after 1064 nm excitation. The sum of the fluorescence of the two individual dyes, **R** and **Cy** (Fig. 3b,c), is identical (within the noise limit) to the fluorescence of the three-dye mixture (Fig. 3a) under identical excitation condition at 1064 nm.

In other control experiments, solutions of the single dyes (**R** or **Cy**) were irradiated with focused laser pulses at 1064 nm generating the fluorescence of **R** or **Cy**, which were shown to be caused by two-photon absorption. Figure 4a shows the fluorescence spectra of **R** observed after excitation at 1064 nm. Reduction of the laser power by 50% reduces the fluorescence by approximately 75%. A quadratic power dependence of the fluorescence intensity of **R** was observed at 1064 nm excitation (Fig. 4d), which is consistent with two-photon excitation, whereas

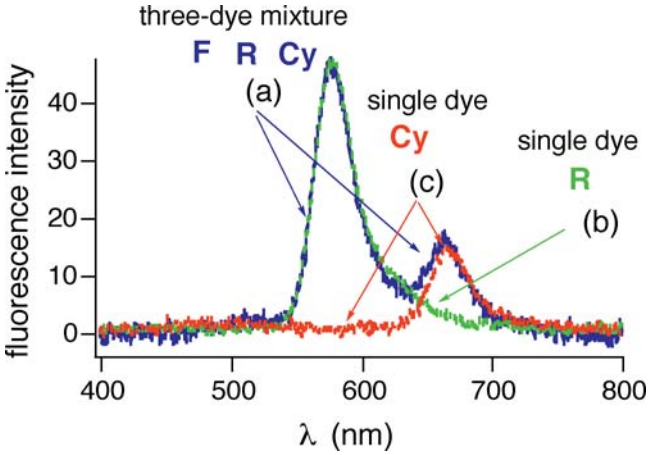


Figure 3. Fluorescence spectra of equimolar solutions of a mixture of **F**, **R**, **Cy** (a) and the single dyes **R** (b) and **Cy** (c) after excitation with focused laser pulses at 1064 nm. The three-dye mixture (a) shows a luminescence spectrum, which is identical (within the noise limit) to the sum of the two fluorescence spectra of the individual dyes (b,c).

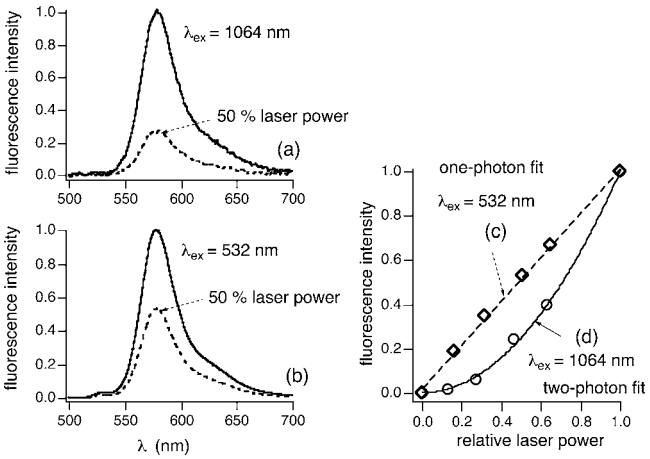


Figure 4. Fluorescence spectra (left) of **R** after excitation at 1064 nm (a) and 532 nm (b) at 100% and 50% laser power. Right: plot of the fluorescence intensity *vs* the relative laser power at 532 nm (c) and 1064 nm (d). Although for one-photon excitations (532 nm) the fluorescence intensity depends linearly on the laser power, the expected quadratic laser power dependence was observed for two-photon absorption (1064 nm), thus confirming two-photon excitation.

a linear power dependence was observed at 532 nm excitation (one-photon excitation) (Fig. 4c).

CONCLUSIONS

The trifluorophore-labeled DNA, **F**-**3**–**R**-**7**–**Cy**-**13**, shows a unique fluorescence signature after excitation with focused laser beams at 1064 nm. This fluorescence is caused by two-photon absorption of **R** and **Cy**, followed by fluorescence energy transfer from **R** to **Cy**. No fluorescence of **F** was observed. The fluorescence signature after two-photon absorption is distinguishable from the three fluorescence signatures after one-photon excitations at 488, 532 and 633 nm (2). This allows for a multi-dimensional analysis of the CFET tags, which increases the probability of true readings in multiplex genetic analysis.

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