

Fluorene-based fluorescent probes with high two-photon action cross-sections for biological multiphoton imaging applications

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1 Introduction

Two-photon excitation fluorescence microscopy (TPM), first demonstrated by Denk et al.¹ in 1990, is based on the condition of a fluorophore to simultaneously absorb two lower energy photons in a single quantum event to induce an electronic excitation that is normally accomplished by a single higher energy photon.² The advantages of TPM have been de-

Abstract. Two-photon fluorescence microscopy is a powerful tool for the study of dynamic cellular processes and live-cell imaging. Many commercially available fluorescent probes have been used in multiphoton-based imaging studies despite exhibiting relatively low two-photon absorption cross-section values in the tunability range of ultrafast Ti:sapphire lasers commonly used in multiphoton microscopy imaging. Furthermore, available fluorophores may be plagued with low fluorescence quantum yield and/or photoinstability (i.e., photobleaching) on exposure to the high peak power and photon density provided by the ultrafast laser source. To address the demand for better performing dyes, we prepare fluorophores tailored for multiphoton imaging. These fluorophores are based on the fluorene ring system, known to exhibit high fluorescence quantum yield (>0.7) and high photostability. Furthermore, an amine-reactive fluorescent probe for the covalent attachment onto amine-containing biomolecules is also prepared. Epi-fluorescence and two-photon fluorescence microscopy images of H9c2 rat cardiomyoblasts stained with an efficient two-photon absorbing fluorene fluorophore is demonstrated. Additionally, single-photon spectral characteristics of the amine-reactive fluorophore, as well as the two-photon absorption cross sections of its model adduct in solution, and spectral characterization of a bovine serum albumin (BSA) as a model bioconjugate are presented. © 2005 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2104528]

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scribed in detail elsewhere,³⁻⁵ and are inherently characterized by the high spatial localization of this excitation event via the quadratic relationship between the excitation and fluorescence intensity. Practically, this means fluorescence occurs only at the focal volume, and as near-IR laser irradiation is used as the excitation source, deeper imaging into optically thick tissue, with spatially restricted photobleaching and phototoxicity of the imaging specimen are achieved. The tenets of two-photon absorption (2PA) enable investigations of complex

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biological problems and experiments on living samples not possible with other imaging techniques, and highlighted examples of its use in the biological research areas have been noted.⁶⁻⁸

Two-photon and higher multiphoton microscopies, utilizing judicious choices of optical probes, have yielded sophisticated and unparalleled imaging methods and techniques relative to what is achievable from linear fluorescence imaging methods. In the case of laser-scanning two-photon excited fluorescence microscopy, selecting the optimal fluorophore is critical, and databases of the 2PA and emission spectra for commonly used fluorophores and bio indicators have been generated to facilitate the selection process.⁹⁻¹³ Many of these compounds are conventional UV-excitable fluorophores, exhibiting low 2PA cross sections (δ), of the order of ~ 10 GM units* with a few exhibiting ~ 100 GM units. The δ parameter is an indicator of the 2PA efficiency, and only recently has research been reported on the design and development of very efficient 2PA dyes possessing between 100 and several thousand GM units.¹⁴⁻¹⁹ A more salient feature of a fluorophore for its use in TPM is its two-photon excited fluorescence action cross section $\delta\eta$, the product of the 2PA cross section and fluorescence quantum yield η . Usually expressed in GM units, the action cross sections of these dyes are typically lower than the 2PA cross section. Higher values of $\delta\eta$ enable detection of lower dye concentrations with reduced laser power required for imaging, resulting in improved signal collection due to suppressed autofluorescence and less phototoxic effects on the sample. Hence, synthetic efforts focused on preparing new nonlinear optical probes specifically engineered to exhibit higher $\delta\eta$ are expected to outperform standard fluorophores currently in use for TPM imaging.

Until recently, optimization of nonlinear optical probes for multiphoton imaging applications have focused mainly on hydrophobic organic compounds studied in organic solvents.¹⁴⁻¹⁹ More current efforts are directed toward developing and evaluating analogous compounds with greater compatibility or relevance to biological environments, such as increasing hydrophilicity^{20,21} as well as specificity, for which some examples²²⁻²⁸ are important cellular ions such as Ca^{2+} and Mg^{+2} , zinc, and H^+ . However, identification and availability of optimized 2PA fluorophores specifically tailored for direct labeling of biomolecules for two-photon induced fluorescence imaging studies are limited.^{29,30} Currently, amine-reactive fluorescent probes exhibiting high 2PA cross sections and sufficient action cross sections, specifically used to covalently label biomolecules are rare and appear to be limited to dipyrrometheneboron difluoride dyes.³¹ Therefore, the necessity to incorporate efficient reactive 2PA fluorophores with high action cross sections for covalent attachment onto biomolecules within the fluorophore design strategy seems timely and fulfills an appropriate requirement that coincides with increasing usage of two-photon excitation fluorescence imaging methods and techniques in the life sciences.

Previous studies from our laboratory have reported on the design and development of fluorene-based organic dyes with very efficient 2PA and fluorescence emission properties.^{14,30} The fluorene ring is a π -conjugated system that enables facile

synthetic manipulation, yielding dyes with tailored spectral and solubilizing properties. Two-photon excited fluorescence microscopy images of a well-characterized¹⁴ 2PA fluorene dye (**1**, Fig. 1 in Sec. 3.1) staining fixed rat cardiomyoblasts was demonstrated and lends credence to our motivation toward developing fluorene-based reagents for multiphoton bioimaging applications. Efforts directed toward preparing, in particular, reactive fluorescent reagents have been initiated with the synthesis of an amine-reactive tag (**3**, Fig. 5 in Sec. 3.2) for labeling, e.g., lysine residues on proteins. A model probe-adduct (**4**) exhibiting high fluorescence quantum yield ($\text{QY} = 0.74$) was also prepared by reacting (**2**) with *n*-butylamine to test its reactivity as an amine-reactive fluorescent label and its spectroscopic and labeling properties have been determined. Finally, a model protein bioconjugate (**5**) was prepared with the reactive fluorophore (**3**) and bovine serum albumin (BSA). Initial demonstration of the fluorenyl tag **3**, as an amine-reactive 2PA fluorophore, is part of our continuing program toward developing more hydrophilic fluorene derivatives with higher action cross sections.

2 Experiments

2.1 Materials, Methods, and Instruments

All solvents and reagents were used as obtained from commercial sources unless specified. ¹H nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury-300 NMR (300-MHz) spectrometer using tetramethylsilane (TMS) as the internal standard. ¹³C NMR spectra were recorded on the same spectrometer (75 MHz) using the carbon signal of the deuterated solvent as the internal standard. Chemical shifts (δ) are reported in parts per million (ppm). Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, Georgia). Fourier-transformed IR (FT-IR) spectra were recorded on a Perkin-Elmer Spectrum One spectrometer. UV to visible spectra were recorded on an Agilent 8453 spectrophotometer using standard 1-cm-path-length cuvettes. Steady state and fluorescent lifetime measurements were performed on a PTI Quantamaster spectrofluorometer. Fluorescence QY calculations were determined using a relative measurement³³ (Rhodamine 6G in ethanol). Two-photon induced fluorescence excitation spectrum of the dye-adduct **4** was performed using the two-photon fluorescence (TPF) method as described in detail in Ref. 32. Bright field transmission and epi-fluorescence microscope images of H9c2 cells, stained with a well-characterized 2PA dye **1**, were collected on a Nikon Eclipse E600 upright microscope. Two-photon excited fluorescence microscopy images of the same H9c2 cells were performed on a modified Olympus IX 70 inverted microscope and Fluoview laser scanning unit accommodating a 10-W Verdi pumping a Ti:sapphire crystal of a Mira 900 (Coherent, Ref. 19).

2.2 Glutaraldehyde Fixation of H9c2 Rat Cardiomyocytes Stained with a 2PA Fluorophore 1

H9c2 rat cardiomyoblast cells [American type culture collection (ATCC) CRL-1446] were plated in a four-well Lab-Tek II chamber slide (Fisher Scientific) and kept in a humidified atmosphere of 5% CO_2 at 37°C 24 h prior to dye staining. The

*The 2PA cross section δ is usually expressed in GM (Goppert-Mayer) units, where 1 GM equals $1 \times 10^{-50} \text{ cm}^4 \text{ s photon}^{-1} \text{ molecule}^{-1}$.

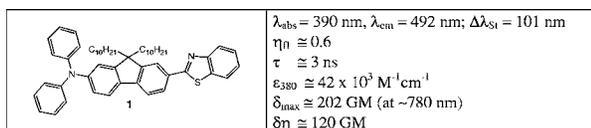


Fig. 1 Structure of a well-characterized 2PA fluorophore and its photophysical properties: λ_{abs} , λ_{em} , $\Delta\lambda_{\text{St}}$ and ϵ are linear absorption, steady state fluorescence emission, Stokes shift, and molar absorptivity, respectively; η_{fl} , and τ are fluorescence quantum yield and lifetime, respectively; δ_{max} and $\delta\eta$ are two-photon cross section and action cross section, respectively.

cells were in Dulbecco's modification of Eagle's medium (DMEM) (Mediatech, Inc.) supplemented with 10% fetal bovine serum (ATCC), 4-mM L-glutamine (Mediatech), and 100 $\mu\text{g}/\text{mL}$ of penicillin-streptomycin (Mediatech) (complete medium). A glutaraldehyde fixation was followed as adapted from protocols presented by Bacallao and Stelzer.³⁴ Briefly, cells were washed with phosphate buffer saline (PBS, pH 7.2, Gibco) to remove growth medium. Cultures were fixed with 0.3% glutaraldehyde (Fisher Scientific) solution in PBS at room temperature, and their cell membranes permeabilized with a 1% Triton-X100 (Sigma) in PBS solution. The detergent was removed with PBS washings, treated with freshly prepared solution of aqueous NaBH_4 solution (1 mg/mL, Aldrich), followed by a brief rinse with 0.1% Triton-X100 in PBS (PBST). An aliquot of compound **1** (10 μL of $4.8 \times 10^{-4} \text{ M}$) dissolved in anhydrous DMSO was delivered to the fixed cells in PBST solution. Details of the synthesis, linear and nonlinear characterization of compound **1** can be found in Refs. 14 and 32. One of the wells did not receive any dye as a control. Cells were washed with PBS ($\times 4$) followed by ddH_2O , and mounted in ProLong Gold (Molecular Probes) mounting medium, cover slipped and sealed.

2.3 Synthesis of Amine-Reactive Fluorene Probe (3), [2-(9,9-didecyl-7-isothiocyanato-fluorenyl)benzothiazole]

The synthesis of the reactive probe was prepared following a literature procedure.³⁵ Briefly, compound **2** (0.48 g, 0.807 mmol), previously prepared,¹⁴ was dissolved in CHCl_3 to which CaCO_3 (0.21 g, 2.11 mmol) dissolved in H_2O was added the mixture. Thiophosgene (0.068 mL, 0.892 mmol) was added dropwise to the vigorously stirring mixture in an ice bath. After ~ 10 min the starting material was completely consumed as determined via TLC (silica, 2:1 hexanes/ CH_2Cl_2), and appeared to have gone to near quantitative conversion. After an additional 20 min, 10% HCl_{aq} was added until no gas generation was observed. The reaction mixture was poured into H_2O , extracted with CH_2Cl_2 , dried over MgSO_4 , and on filtration and concentration, resulted in an orange oil. Purification was accomplished via flash chromatography (silica, 2:1 hexanes/ CH_2Cl_2 eluent). A pale yellow viscous oil was isolated (91% yield). The FT-IR spectrum of isolated compound revealed the characteristic strong —NCS stretch at 2093 cm^{-1} ; no signal at 3600 cm^{-1} from the —NH_2 group was observed. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ : 8.01 (s, 1H, ArH), 8.07 (s, 1H, ArH), 8.04, 8.01 (dd, 1H, ArH), 7.90 (d, 1H, ArH), 7.72 (d, 1H, ArH), 7.68 (d, 1H,

ArH), 7.49 (t, 1H, ArH), 7.38 (t, 1H, ArH), 7.23 (d, 1H, ArH), 7.20 (d, 1H, ArH). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ : 166.0, 154.0, 152.9, 151.5, 142.4, 139.1, 134.7, 134.5, 132.6, 130.1, 127.1, 126.2, 125.0, 124.7, 122.9, 121.4, 121.3, 120.9, 120.3, and 120.2. Calculated analyses for $\text{C}_{41}\text{H}_{52}\text{N}_2\text{S}_2$ (637.0): C, 77.31; H, 8.23; N 4.40. Found: C, 77.11; H, 8.45; N, 4.31.

2.4 Synthesis of Dye Adduct (4), 1-(7-benzothiazol-9,9-didecyl-fluorenyl)-3-butylthiourea

A mixture of reactive reagent **3** (0.85 g, 1.43 mmol) and *n*-butylamine (1.0 mL) was stirred at room temperature for 30 min. The excess butylamine was removed *in vacuo*, and the residue was purified by column chromatography using $\text{CH}_2\text{Cl}_2/\text{hexane}$ [2/1(v/v)] as eluent. Solvent removal and recrystallization from *n*-hexane afforded 0.67 g of white solid (66% yield). Melting point (m.p.) 121 to 122°C; $^1\text{H NMR}$ (250 MHz, $\text{CD}_2\text{Cl}_2\text{—D}_2$) δ : 7.99 (m, 2H), 7.85 (d, $J=6.0$ Hz, 1H), 7.72 (d, $J=6.5$ Hz, 2H), 7.58 (s, 1H), 7.42 (t, $J=7.0$ Hz, 2H), 7.31 (t, $J=6.9$ Hz, 2H), 7.13 (m, 2H), 5.96 (s, 1H), 3.53 (m, 2H), 1.96 (m, 4H), 1.46 (m, 2H), 1.28 (m, 2H), 1.01 (m, 28H), 0.85 (t, $J=3.9$ Hz, 3H), 0.73 (t, $J=3.8$ Hz, 6H), 0.56 (m, 4H). Calculated analyses for $\text{C}_{45}\text{H}_{63}\text{N}_3\text{S}_2$ (710.13): C, 76.11; H, 8.94; N, 5.92. Found: C, 76.28; H, 9.19; N, 5.90.

2.5 Preparation and Characterization of the BSA-Fluorene Model Bioconjugate (5)

Bovine serum albumin (Sigma, Fraction V, $\sim 99\%$) was used as received for conjugation. The bioconjugation reaction was adapted as presented in Ref. 36. Briefly, a stock solution of BSA (1 mg/mL) in freshly prepared NaHCO_2 (0.1 M, pH 9) solution was used. A stock solution of reactive tag (**3**) dissolved in anhydrous DMSO ($5.23 \times 10^{-3} \text{ M}$) was freshly prepared and aliquots were slowly added dropwise into a gently stirring BSA solution (1 mL of stock). The concentration of the probe solution was varied such that a 1:10 and a 1:5 mol ratio of protein to probe were prepared to establish a degree of labeling (DOL) for the probe. The reaction was covered from light and allowed to stir at room temperature for 1 to 2 h, after which the mixture was passed through a Sephadex G-25 fine column (12 cm length, Fluka) equilibrated in and eluted with PBS (pH 7.2; Gibco). Fractions containing the bioconjugate were identified spectrophotometrically by monitoring both the protein fraction at 280 nm and the dye at 360 nm. The BSA protein concentration was approximated in the BSA-fluorene bioconjugate via a BSA calibration curve using the Beer-Lambert law. The molar absorptivity (ϵ_{278}) of BSA in PBS (pH 7.2) was determined to be $39.4 \times 10^3 \text{ Lmol}^{-1} \text{ cm}^{-1}$ and corresponds well to the literature value³⁷ of $\epsilon_{278}=44 \times 10^3 \text{ Lmol}^{-1} \text{ cm}^{-1}$ (in 0.1 M Tris-Cl, pH 8.2). Calculation of the DOL was approximated from the product information page provided by Molecular Probes on amine-reactive probes and from Ref. 36.

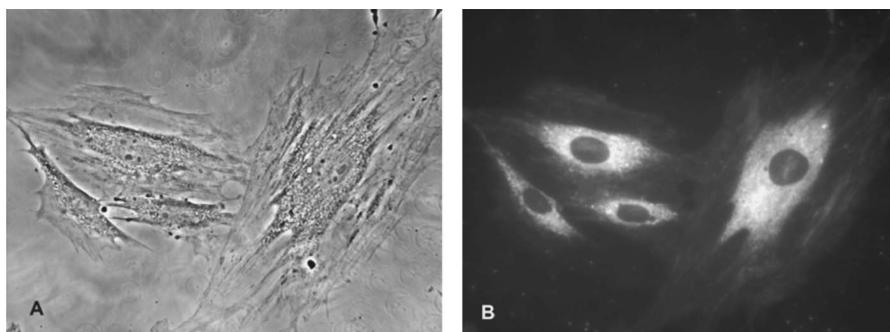


Fig. 2 (A) Bright field transmission and (B) epi-fluorescent microscopy images of H9c2 cells stained with a 2PA dye 1.

3 Results and Discussion

3.1 Epi-Fluorescence and Two-Photon Excited Fluorescence Microscopy Images of H9c2 Cells Stained with a Two-Photon Absorbing Dye 1

Compound **1** is a well-characterized fluorophore that exhibits relatively high 2PA cross sections in the excitation range of a pulsed Ti:sapphire laser output. Figure 1 shows the structure of compound **1** along with pertinent photophysical data characterized in acetone. Note that this compound was studied in a range of solvents of varying polarity, from hexanes to acetonitrile and acetone, and exhibits solvatochromic effects that influence its photophysical properties. Details of solvent effects on both its linear and nonlinear optical properties are presented in Ref. 32. Hence, the properties presented are indications of typical values to be expected from the highly photostable derivative.¹⁴ Note that additional fluorene derivatives with much higher cross sections, and hence, action cross sections, have been prepared,¹⁹ and choice of fluorene **1** was selected as a proof-of-principle demonstration that fluorene derivatives can be used in microscopy imaging of biological samples.

The utility of fluorene **1**, and of additional fluorene-based derivatives, as efficient 2PA biological fluorophores was demonstrated by staining fixed H9c2 rat cardiomyoblast cells.

Bright field transmission and epi-fluorescence microscope images [DAPI filter set, 40 \times , numerical aperture (NA) 0.75] of the stained cells are shown in Fig. 2. Furthermore, fluorene **1** did not undergo noticeable photobleaching during continuous exposure to the UV excitation light. No fluorescence was observed in the controls without any fluorophore (image not shown). Additionally, fluorescence was observed predominantly from the cytoplasmic region of the cells, with the nucleus clearly outlined, indicating potentially preferential staining of this fluorophore for cytoplasmic components.

TPM images of the same fluorene **1**-stained cells were collected on a modified Olympus IX-70 inverted microscope and Fluoview laser scanning confocal unit with a Ti:sapphire laser output from 740 to 830 nm (125 fs FWHM, 76 MHz repetition rate, \sim 25 mW, 40 \times , NA 0.85). The control cells that did not receive any fluorophore showed modest autofluorescence on 800 nm fs excitation, as shown in Fig. 3(C), while the fluorophore-stained cells [Fig. 3(D)] revealed higher contrast and greater signal under the same excitation and power exposure as the control. Two-photon induced fluorescence was observed predominantly from the cytoplasmic region, consistent with the images collected from epi-fluorescence images.

A direct quadratic dependency of fluorescence intensity on excitation power to verify a 2PA process was difficult to de-

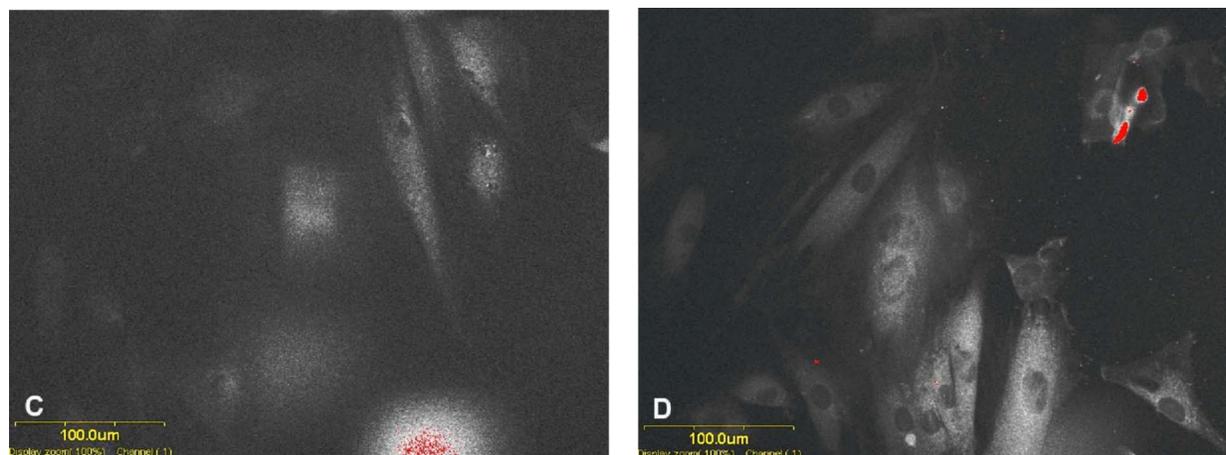


Fig. 3 TPM images of (C) blank containing no fluorophore and exhibiting some autofluorescence and (D) cells stained with fluorene **1** upon 800 nm fs excitation. Red spots demark signal saturation.

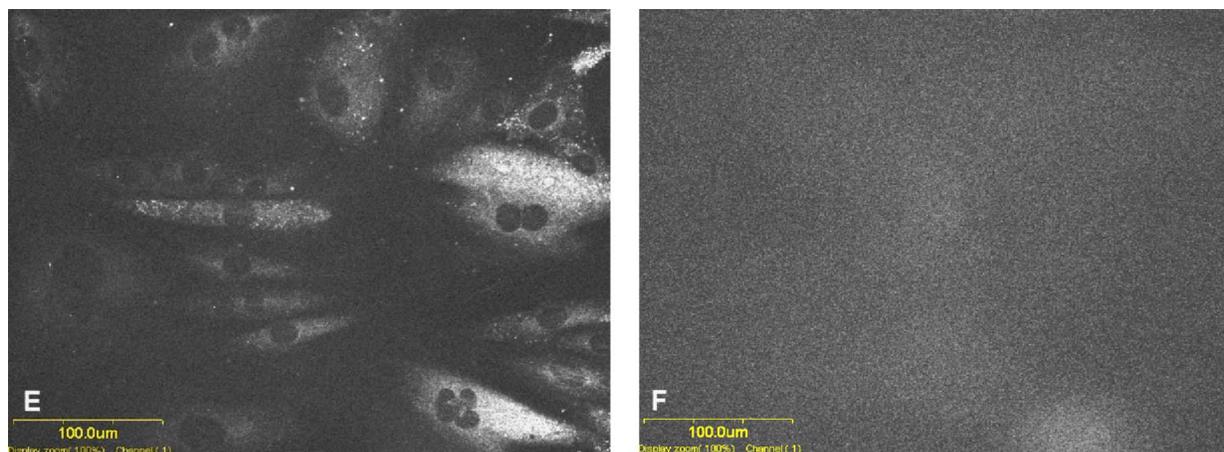


Fig. 4 TPM images of H9c2 cells stained with fluorene **1** under (E) mode-locked and (F) non mode-locked Ti:sapphire irradiation conditions.

termine in the highly scattering sample. Hence, assertion of multiphoton excited fluorescence was verified by imaging the same sample area and image plane using the same average power under mode-locked (ML) and non-mode-locked (cw) conditions. A fluorescent image under ML conditions [Fig. 4(E)] was clearly generated, while no image was obtained under cw conditions [Fig. 4(F)], and while higher order processes such as three-photon absorption may be involved, spectroscopic data suggest a two-photon process in the excitation range used (740 to 830 nm) would be dominant for compound **1**. Hence, two-photon excited fluorescence images of H9c2 cells stained with fluorene **1** was demonstrated, providing strong motivation for the development of fluorene-based reactive reagents and probes for multiphoton bioimaging applications.

3.2 Characterization of a Model Adduct (4) Prepared with an Amine-Reactive Fluorenyl Tag (3)

Details of the synthesis and characterization of the fluorenyl derivative (**2**) was previously reported.¹⁴ Compound (**3**) was prepared as an amine-reactive fluorescent tag (Fig. 5), and contains the isothiocyanate functionality for covalent bond formation with primary amine groups present on protein molecules. A model dye-adduct (**4**) was also prepared by reacting (**3**) with *n*-butylamine to test its reactivity as an amine-reactive fluorescent label. Preparation of the model adduct allowed for facile single- and two-photon spectroscopic characterizations that more closely resembles the bioconjugate than that of the reactive fluorophore alone.

The normalized UV to visible absorption and steady state fluorescence emission spectra of the free reactive tag (**3**) and the dye adduct (**4**) in DMSO are shown in Fig. 6. The free reactive fluorophore exhibits two absorption maxima at 357 and 375 nm, along with two emission maxima at 384 and 404 nm. The dye adduct instead exhibits a single absorption maximum at 363 nm with an emission maximum at 403 nm, and is well resolved from that of its absorption spectrum, with minimal spectral overlap. The fluorescence quantum yield of the reactive reagent in DMSO was 0.02, virtually nonfluorescent, while that of the dye adduct in DMSO increased significantly to 0.74, indicating the fluorescence of the reactive tag is restored upon conjugation to a biomolecule.

The 2PA cross section for the dye adduct in DMSO was obtained using the two-photon induced fluorescence (2PF) method under femtosecond, near-IR irradiation conditions. In the 2PF method a strong, tunable pump beam excites the chromophore via 2PA, and the subsequent induced fluorescence is monitored as a function of the excitation wavelength. The 2PF results obtained for the fluorophore under investigation are calibrated against well-known reference standards. Furthermore, the quadratic dependence of 2PF on the pump irradiance is verified for multiple excitation wavelengths. A more detailed explanation of the experimental details can be found in Ref. 32.

The linear absorption and fluorescence emission spectra of the model adduct (line and dashed profiles, respectively) and the two-photon induced fluorescence excitation spectrum measured at wavelengths twice that of the linear absorption (data points) are displayed in Fig. 7. The 2PF measurements of the model adduct were performed in DMSO (1.6×10^{-3} M), and exhibited a 2PA cross section of ~ 25 GM units at the linear absorption maximum of 370 nm. With its fluorescence quantum yield of ~ 0.74 , the action cross section for the model adduct is ~ 19 GM units. This is higher than most commonly used amine-reactive dyes. More importantly, additional hydrophilic fluorenyl derivatives, analogous in structure to compound **1**, are being developed and are expected to exhibit higher action cross sections.

Interestingly, while the linear absorption spectrum for the compound does not display any significant absorption at the shorter wavelengths, the value of the 2PA cross section increases, possibly accessing higher excited-state transitions of the fluorophore. To ensure the dye adduct is undergoing two-photon absorption, a log-log plot of the femtosecond pump power to that of the integrated fluorescence was constructed. As we can see from Fig. 8, the slopes from the measurements (inset of graph) confirm the quadratic dependence of fluorescence obtained from a two-photon absorption process.

3.3 Characterization of a Model Bioconjugate (5) Prepared with an Amine-Reactive Fluorenyl Tag (3)

The amine-reactive fluorenyl reagent (**3**) was used to label BSA protein, a model biomolecule. The use of BSA, an inex-

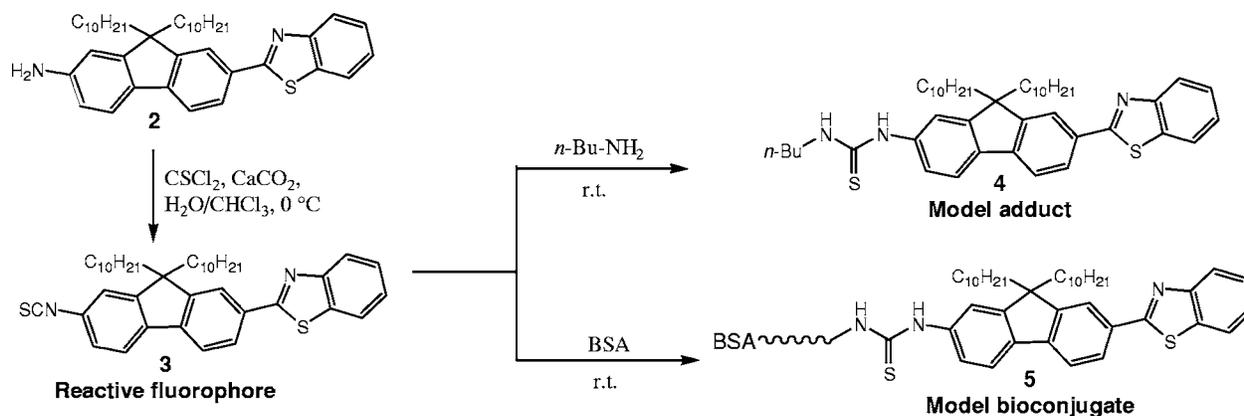


Fig. 5 Preparation of the model adduct (4) and bioconjugate (5) with the amine-reactive fluorenyl reagent (3).

pensive protein that has been extensively characterized, is ideal for establishing optimal conditions to obtain a model bioconjugate, facilitating subsequent spectroscopic characterization. The isothiocyanate functionality reacts with aliphatic amine groups, including the *N*-terminus of proteins and the ϵ -amino groups of lysines (pKa \approx 10.5). A typical protocol for conjugation was followed in an amine-free buffer under slightly basic pH (pH=9.0) conditions. The conjugate was identified spectrophotometrically and its steady state fluorescence emission spectra subsequently obtained. Two different molar ratios of the reactive dye to protein under different reaction times were performed to assess the reactivity of the dye for its DOL (Table 1). The DOL was estimated using standard equations obtained from Ref. 24. The DOL is a key parameter to establish as overlabeling of a fluorescent tag may interfere with the biological activity of a particular protein. Hence, a DOL value of \sim 2.2 to 3.4 was obtained with the amine-reactive tag, a typical range for amine-reactive probes.

The normalized absorption and steady state fluorescence emission spectra of the BSA-dye conjugate (5) in PBS buffer (pH 7.2) are shown in Fig. 9. For reference, the absorption

spectrum of the free BSA protein in PBS solution is also shown. The conjugate displays absorption peaks corresponding to that of the BSA protein in the shorter wavelength range ($\lambda_{\text{max}}=280$ nm), as well as that of the fluorescent tag in the longer absorption range ($\lambda_{\text{maxima}}=360$ and 380 nm). The fluorescence emission of the bioconjugate is broad and exhibits an appreciable Stokes shift. A bathochromic shift in the fluorescence emission was observed in the BSA-dye conjugate, relative to that of the free reactive fluorophore. Similar to the broadening observed in the absorption profile, the fluorescence emission of the model conjugate was also broader than that of the free fluorophore. The observed Stokes shift in the free dye was about 45 nm, while that of the BSA-dye conjugate was much greater (Stokes shift=73 nm on $\lambda_{\text{ex}}=360$ nm, and 53 nm on $\lambda_{\text{ex}}=380$ nm). The fluorescence emission profile of the BSA-dye conjugate on excitation at $\lambda_{\text{ex}}=360$ nm and $\lambda_{\text{ex}}=380$ nm yielded similar fluorescence intensities. The

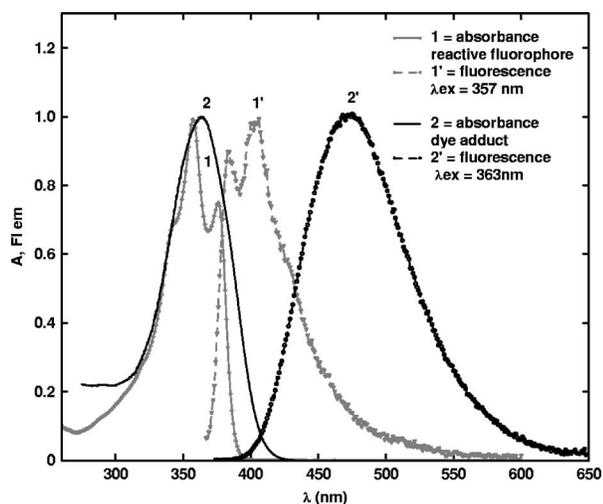


Fig. 6 Normalized UV to visible absorbance (1 and 2) and fluorescence emission (1' and 2'; excitation line indicated) spectra of the amine-reactive tag (3) and the dye adduct (4) in DMSO.

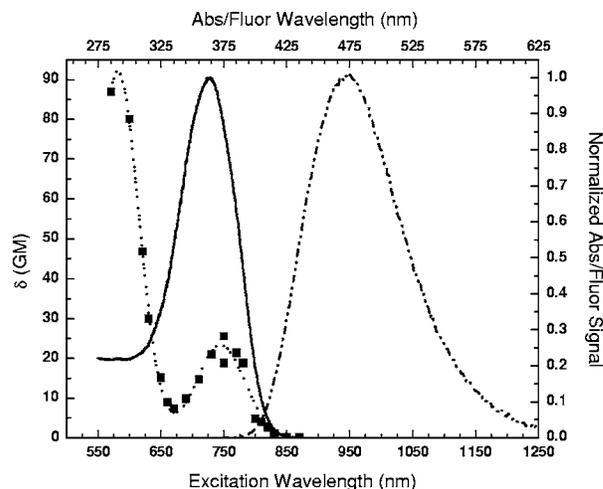


Fig. 7 Linear and nonlinear spectra of dye adduct in DMSO. The solid line is the normalized one-photon absorption spectrum; the dashed line is the normalized one-photon fluorescence spectrum. The two-photon induced fluorescence excitation spectrum is represented by filled symbols and the dotted line is a two-peaked Gaussian fitting function. The γ axis (left) denotes 2PA cross sections in GM units (1×10^{-50} cm⁴ s photon⁻¹ molecule⁻¹) and the x axis (bottom) represents the two-photon excitation wavelength.

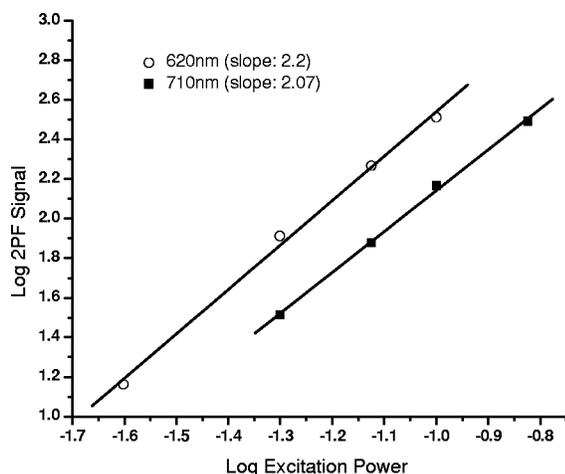


Fig. 8 Log-log plot of two-photon induced fluorescence signal versus excitation power at two different excitation wavelengths extracted from the data shown in Fig. 7. The solid lines are linear fitting functions whose slopes are indicated in the inset of the graph. The slopes show a quadratic dependence indicative of a two-photon induced process.

spectral profile of the model conjugate indicates the electronic property of the fluorenyl tag is perturbed upon binding and may additionally be affected by the proximity of the protein.

4 Conclusion

Development of fluorene-based reactive probes and tags for multiphoton bioimaging applications arises out of our systematic studies on the molecular structure to nonlinear optical property relationships for a wide range of fluorenyl derivatives. These derivatives exhibit desirable spectral properties, such as high photostability, high fluorescence quantum yields, high two-photon absorption cross sections, and therefore, action cross sections, over the tunability range of commercial Ti:sapphire lasers typically utilized in multiphoton imaging

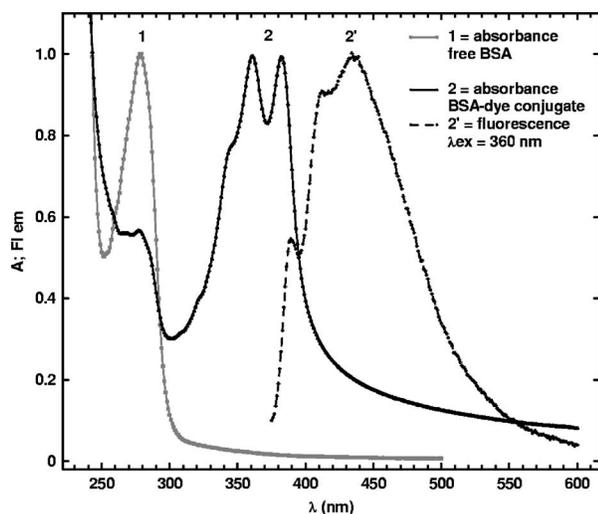


Fig. 9 Normalized absorption spectra of the free BSA protein and BSA-dye conjugate (1 and 2) and steady state fluorescence emission spectrum of the BSA-dye conjugate (2').

Table 1 Variable DOL obtained on altering the molar ratios of the reactive tag to BSA protein.

BSA	Reactive Fluorophore	Dye: Protein mol Ratio	Reaction Time	DOL (mol dye: mol protein)
1.53×10^{-8} mol	1.53×10^{-7} mol	10:1	1 h	3.4
1.53×10^{-8} mol	7.7×10^{-8} mol	5:1	1 h	2.4
1.53×10^{-8} mol	7.7×10^{-8} mol	5:1	2 h	2.2

methods and techniques. Such properties make these fluorenyl derivatives compelling candidates for multiphoton bioimaging applications, and in particular, as probes or tags for direct covalent linkage onto biomolecules.

Demonstration of TPM images of H9c2 cells stained with a well-characterized 2PA fluorophore lends credence to our efforts to further refine fluorene-based derivatives for bioimaging applications. An amine-reactive fluorenyl tag was prepared and shown to effectively label primary amines as evidenced by preparing the model adduct. The model adduct allows for improved spectroscopic characterization. Furthermore, preparation of the model BSA-dye conjugate and its spectroscopic details validate the use of the fluorenyl-base reactive tag (3) to form bioconjugates with protein biomolecules.

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