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A Photostable, pH-Invariant Fluorescein Derivative for Single-Molecule Microscopy

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Abstract We herein report the comprehensive characterization of the spectral and single-photon fluorescence properties of a recently synthesized fluorescein derivative and its biotinylated analog. The fluorophore displays significant increases in photostability compared to the known fluorescein label fluorescein isothiocyanate (FITC), as well as superb pH independence. This fluorescein variant has two readily accessible functional groups (aniline NH_2 and phenol OH) that can be activated or blocked independently and can serve, for instance, as a fluorescent bridge between two different recognition motifs. Excellent single-photon counting fluorescence data demonstrates that it is also a particularly appropriate probe for single-molecule studies of biological interactions.

Keywords Fluorescence spectroscopy · Photostability · Single-molecule biophysics · FRET

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Introduction

Fluorescein is one of the most widely used green-fluorescent labeling dyes in biomedical research due to its favorable spectral properties in aqueous media [1–3]. However, low photostability and steep pH-dependence are considerable drawbacks for biological studies, in particular those making use of single-molecule fluorescence. Depending upon pH, fluorescein can exist in four different prototropic forms (monoanion, dianion, neutral, and cation), which have significantly different absorption/emission properties [4], and this phenomenon is detrimental for fluorescence labelling [5].

One of the most general strategies for using fluorescent probes in biophysical studies involves the synthesis of conjugates with invariant fluorescence properties to environmental factors such as pH, polarity and viscosity. These conjugates are attached to specific sites of biomolecules and used to delineate molecular interactions through techniques such as single-molecule spectroscopy, fluorescence correlation spectroscopy and Förster Resonance Energy Transfer (FRET) [6, 7].

We herein report the comprehensive spectral characterization of 2-(9-(4-acetamido-2-(ethoxycarbonyl)phenyl)-3-oxo-3*H*-xanthen-6-yloxy)acetic acid (compound **5** in Fig. 1, see below), a recently reported fluorescein derivative [8]. Our studies show that **5** exhibits remarkable pH invariance and photostability. Moreover, the molecule is a bright fluorophore, exhibiting both high absorption cross section and high fluorescence quantum yield. In addition, **5** was tested in single-molecule experiments under continuous illumination and lasted for several seconds or minutes. Significantly, fluorescein derivative **5** has two readily accessible functional groups (aniline-NH_2 and phenol-OH) that can be activated or blocked independently and can serve,

for instance, as a fluorescent bridge between two different recognition motifs (bivalent inhibitor). **5** is compared with fluorescein isothiocyanate (FITC), a known fluorescein derivative commonly used in biophysical fluorescence studies [1, 2]. The superior spectral properties and versatility of compound **5** make it an attractive yet inexpensive probe for a wide range of fluorescence microscopy studies.

Materials and methods

Synthesis The fluorescein derivative **5** was synthesized in a similar manner to that described previously [8], with minor modifications. Briefly, as shown in Fig. 1, the carboxylic acid of 5-aminofluorescein (**1**) was protected as its ethyl ester by refluxing with ethanol in the presence of thionyl chloride to furnish **2** in quantitative yield, and then subsequent chemoselective alkylation of the phenolic group with *tert*-butyl bromoacetate afforded **3**. Acetylation of the amino group of **3** followed by TFA-mediated deprotection of the *tert*-butyl ester yielded compound **5**, or alternatively **3** was condensed with biotin, employing HBTU as the coupling reagent, to furnish **6**, which was then deprotected as before to deliver **7**. Compounds **5** and **7** were spectrally characterized in this study.

*NMR Characterization of 2-(9-(4-acetamido-2-(ethoxycarbonyl)phenyl)-3-oxo-3H-xanthen-6-yloxy)acetic acid (**5**)* δ_H (400 MHz, *d*-$CDCl_3$) 0.86 (t, *J*=7.2 Hz, 3H, $C\underline{H}_3CH_2$), 2.13 (s, 3H, $C\underline{H}_3CO$), 3.92 – 4.00 (m, 2H, $C\underline{H}_2CH_3$), 4.93 (s, 2H, $OC\underline{H}_2CO$), 6.40 (d, *J*=1.9 Hz, 1H, CH (Ar)), 6.52 (dd, *J*=9.6 and 1.9 Hz, 1H, CH (Ar)), 6.96 – 7.04 (m, 3H, 3 CH (Ar)), 7.27 (d, *J*=2.2 Hz, 1H, CH (Ar)), 7.42 (d, *J*=8.4 Hz, 1H, CH (Ar)), 8.08 (dd, *J*=8.4 and 2.2 Hz, 1H, CH (Ar)), 8.43 (d, *J*=2.2 Hz, 1H, CH (Ar)), 10.48 (s, 1H, $COO\underline{H}$). NMR data agrees with previously published results [8].

Steady-state spectroscopy Absorption measurements were performed in a Cary14 spectrophotometer (On-Line Instrument Systems, Bogart, GA). The absorption spectra were corrected for cuvette and solvent absorbance at each pH. Different pH buffers were prepared by mixing different amounts of sodium phosphate monobasic and dibasic followed by fine adjustement using small amounts of concentrated sodium hydroxide or hydrogen chloride solutions. Emission spectra at each pH were recorded in a Quanta-Master fluorescence spectrometer (Photon Technology International, Birmingham, NJ) with 3 nm bandwidth. The fluorescence quantum yields of **5** and FITC were obtained by comparison to a NIST standard fluorescein sample having a yield φ=0.93 in 0.1 M NaOH (R14782, Invitrogen Canada). Emission spectra were measured for each sample under high dilution, to insure an identical, low absorbance at the excitation wavelength, i.e. ~0.01 at 470 nm. The ratio of integrated fluorescence intensities of any two samples will give the ratio of their fluorescence quantum yield values.

Single-molecule spectroscopy The experiments were performed on a custom-built confocal microscope using a broadly tunable femtosecond laser (Tsunami HP, Spectra Physics, Mountain View, CA) as excitation source. In brief, the laser is frequency-doubled in a nonlinear crystal to obtain a narrow spectrum centered at 480 nm (spectral width~6 nm). The light is then passed through a 1.4 NA/

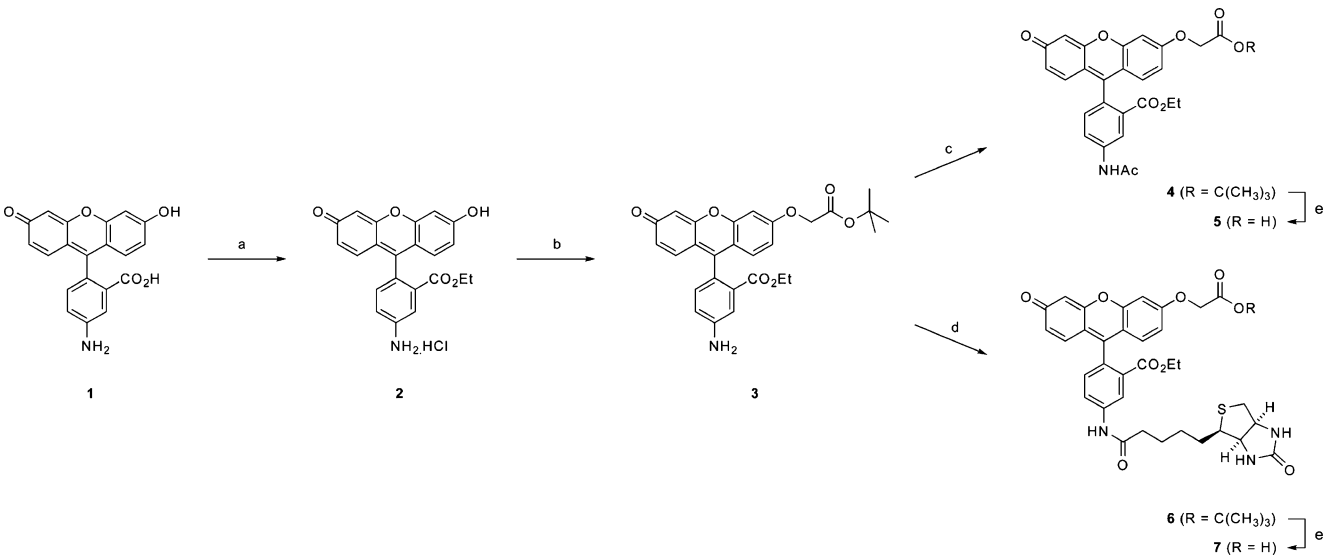

Fig. 1 **a** $SOCl_2$, EtOH, reflux, 24 h, 100%; **b** $BrCH_2CO_2C(CH_3)_3$, K_2CO_3, DMF, rt, 16 h, 74 %; **c** Ac_2O, EtOH, 45°C, 18 h, 77 %; **d** Biotin, HBTU, DIPEA, DMF, rt, 16 h, 38 %; **e** TFA-CH_2Cl_2, 1:1, rt, 3 h, 100%

100x microscope oil objective (Carl Zeiss Canada) and illuminates the sample at intensities of 50–100 W/cm^2. The samples were spin-coated on plasma-cleaned glass coverslips from solutions of 100 pM of dye, 1% w/v poly (methyl-methacrylate) (PMMA) in toluene. The fluorescence is collected through the same objective, spatially and spectrally filtered to remove the resonant scattering and focused onto a sensitive avalanche photodiode (PD5CTC, Optoelectronic Components, Kirkland, Canada). Each time a photon is detected, the detector outputs an electric pulse, and these pulses are counted by a PicoHarp300 module (PicoQuant Gmbh, Germany). The sample is raster scanned in the xy plane by a 3-axis piezo scanner (T225, MadCity Labs, Madison, WI) so that confocal fluorescence images are typically obtained in 10–60 sec for an area of 10×10 microns. The single fluorophores, which correspond to the bright spots in the image, are brought into focus one by one and continuously excited until single-step, irreversible photobleaching occurs. The fluorescence lifetimes were measured by the time-correlated single-photon counting (TCSPC) technique on the same microscope setup. The experiments used diluted dye solutions (50 nM fluorescein in PBS buffer, pH 8) on glass coverslips coated with BSA (bovine serum albumin) to prevent nonspecific adsorption to glass.

Photostability The photobleaching behavior was studied using 1 μM solutions of **5** or FITC at pH 8. A small sample volume was created by punching a 2 mm diameter disc on a 1.2 mm thick plastic membrane that adheres to a microscope coverslide. The slide with the sample on it was mounted on an inverted wide-field fluorescence microscope in which a 0.23 NA/5x air objective was used to deliver the excitation light and to collect the red-shifted fluorescence. The fluorescence signal of the sample was detected by a cooled EMCCD (Electron-Multiplified Charge-Coupled Device) camera (DU-897BV, Andor, South Windsor, CT). The camera was exposed for 10 ms at 1 sec intervals during continuous illumination of the sample. The sample was excited at 473 nm with light from a diode-pumped solid-state laser (Cobolt Blues, MarketTech, Scotts Valley, CA) at intensities in the order of 0.5 W/cm^2.

Results and discussion

The absorption spectrum of **5** at pH 8 (Fig. 2) exhibits two bands of almost identical intensity, at 457 nm and 483 nm. The extinction coefficient of **5** is $\varepsilon_{max}=2.2\times10^4 M^{-1}cm^{-1}$ at 457 nm, smaller than the measured value for FITC ($\varepsilon_{max}=8.1\times10^4$ at 494 nm), but similar to the reported values for 3-O-alkyl-fluorescein-2′-esters [8, 9]. The red-most peak at

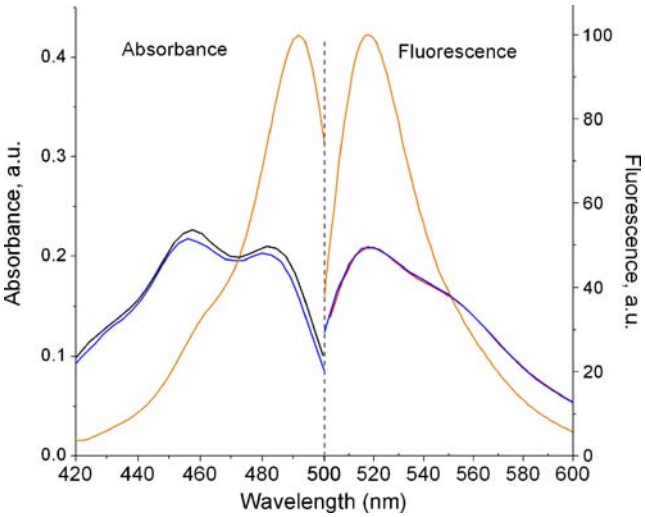

Fig. 2 Absorption and emission spectra of compound **5** (blue) and FITC (orange) at pH 8. Also shown are the absorption spectrum of the biotinylated derivative **7** (black, left panel) and the emission spectrum of **5** upon excitation at 457 nm (red, right panel)

483 nm corresponds to the absorption maximum of FITC at 494 nm. The 457 nm peak corresponds to the absorption shoulder of FITC near 460 nm, which was found to be characteristic of various fluorescein ethers having 2′-esters or 2′-secondary amide residue [8–11]. The emission spectra measured upon excitation at 457 or 483 nm are identical (Fig. 2, right panel), as are the fluorescence decay times (2.83±0.10 ns at pH 8, data not shown). This indicates that the two absorption bands correspond to the same functional group and the molecule is excited to the same electronic level, but to a different vibrational state. The vibrational mode predominant in the absorption spectrum has a frequency of 1178 cm^{-1}, which can be assigned to the CCH bending mode on the xanthene ring [12]. The emission of **5** and FITC both peak close to 520 nm but the former is much broader (75 nm vs. 40 nm). At pH 8, the fluorescence quantum yield of the synthesized derivative was estimated to be ϕ_5=0.22, consistent with the values reported previously for some 3-O-alkyl fluorescein ethers [4, 8, 13, 14], whereas ϕ_{FITC}=0.52, as reported in earlier studies [13, 15, 16].

The broadening of the emission spectrum and the decrease of the fluorescence quantum yield are probably caused by alteration of D_{2h} molecular symmetry of the xanthene moiety [8, 12]. The relatively broader absorption and emission spectra observed, coupled with a larger Stokes shift, may offer better conditions for single molecule detection [17–19]. The scattered excitation light can be more efficiently removed by long-pass edge filters without significantly reducing the fluorescence signal. On the other hand, a broader emission spectrum offers the opportunity for FRET pairing with long-wavelength acceptor probes; with Alexa-647, the Förster distance is estimated to be around 45Å. For compounds **5** and **7** the absorption spectra

are indistinguishable and all other spectral parameters of **7** are virtually identical to those of **5** (data not shown). Therefore we conclude that ligation chemistry (step (d) in Fig. 1) and the presence of the ligand (biotin) do not affect the electronic properties of the fluorophore.

As shown in Fig. 3, the emission spectrum of compound **5** are practically pH-independent, which is not typical for fluorescein dyes. For instance, the spectral properties of FITC change dramatically outside a narrow pH range [15, 20]; the emission spectra of FITC are quite narrow (~40 nm) in basic conditions and become much broader at acidic values (~70 nm at pH<5, data not shown). The overall fluorescence intensity is also pH-independent for compound **5**, as shown in Table 1. The fluorescence quantum yield of **5** is constant at 0.22 for pH values between 4 and 10 and exhibits a minor reduction outside this range. In contrast, the quantum yield of FITC varies noticeably for pH<7 and pH>10: from 0.57 at pH 9 to 0.01 at pH 2.3 and 0.10 at pH 12. An almost identical trend was observed for the variations of the fluorescence lifetime measured at different pH values. Figure 4 shows the fluorescence decay data measured from dye solutions at pH 8. The measurments were repeated at several pH values and the curves obtained were fitted to a monoexponential decay function using a custom-built program based on the Levenberg-Marquardt algorithm. The time constants obtained from the fitting are listed as fluorescence lifetimes in Table 1. The same as for quantum yield, minor lifetime changes occured for **5**, while significant variations of FITC lifetime were observed particularly in the acidic range. As $\phi=\tau_{fl}/\tau_{rad}$, one can conclude that the radiative decay is not affected by pH for either molecule, whereas the non-radiative processes, i.e. internal conversion, are enhanced at extreme pH values for fluorescein and are almost invariant for compound **5**. This is explained by the disruptive influence of proton charges on the xanthene ring in fluorescein, while the only deprotonation site is far away in compound **5** to cause significant alterations of its fluorescence properties.

Table 1 The pH-dependence of fluorescence quantum yield and decay time of the compound **5** and FITC

pH	2.3	4	6	8	9	10	12
ϕ_5	0.18	0.22	0.22	0.22	0.22	0.21	0.18
ϕ_{FITC}	0.01	0.19	0.39	0.52	0.57	0.51	0.11
τ_5(ns)	2.4	2.8	2.8	2.8	2.8	2.8	2.4
τ_{FITC} (ns)	2.4	2.9	3.3	3.7	3.9	3.4	2.5

The fluorescence quantum yields were determined from emission spectra using the comparative method described in the text. The estimation error is ±0.01. The decay times represent the time constants of monoexponential functions used to fit experimental time-resolved data. The errors in estimating the lifetimes are ±0.10 ns.

Optical imaging of biological systems usually requires small amounts of fluorescent probes that are supposed to remain active long enough for studying dynamics for several seconds, minutes or hours. Under such conditions, chemical alteration and irreversible photobleaching of the fluorescent probes become limiting factors for biomedical fluorescence imaging. Therefore, we performed experiments comparing the photostability of compound **5** and FITC by using small volumes of dye solutions under continous and uniform illumination at high laser excitation intensity (see above for details). The fluorescence of each sample was monitored by a CCD camera in a wide-field fluorescence microscope setup and the signal was integrated across the entire field of view. As shown in Fig. 5, the FITC fluorescence decreased rapidly with a characteristic half lifetime of $t_{1/2}$~13 s, in agreement with previous reports [8]. Overall, FITC intensity decreased by 82% in

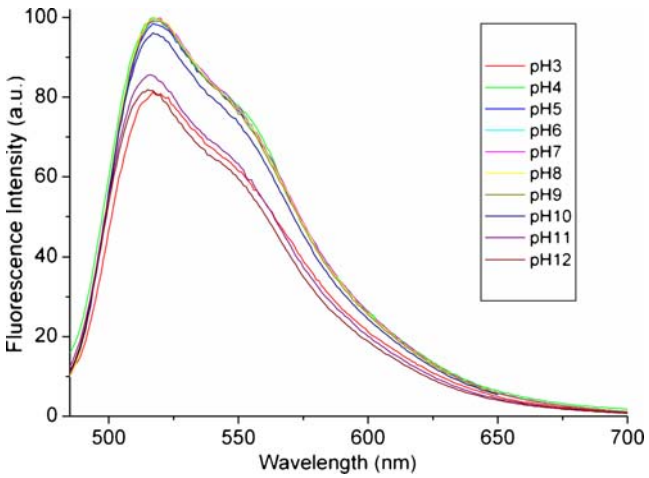

Fig. 3 pH dependence of the emission spectrum of compound **5**. The spectra were normalized by the optical density at the excitation wavelength (470 nm)

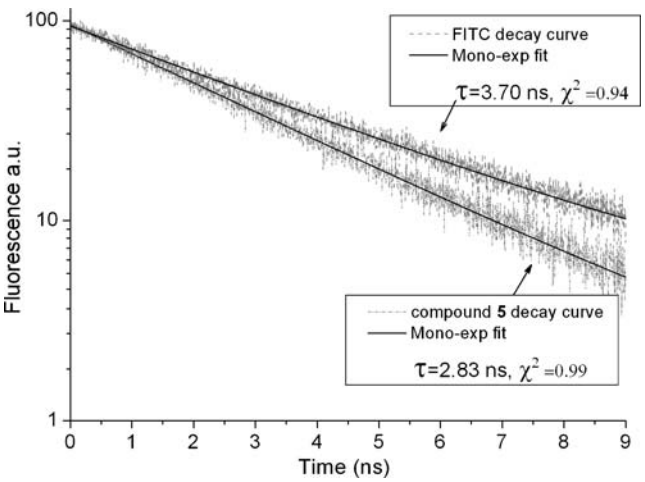

Fig. 4 Nanosecond fluorescence decay data of compound **5** and FITC at pH 8 fitted with mono-exponential functions

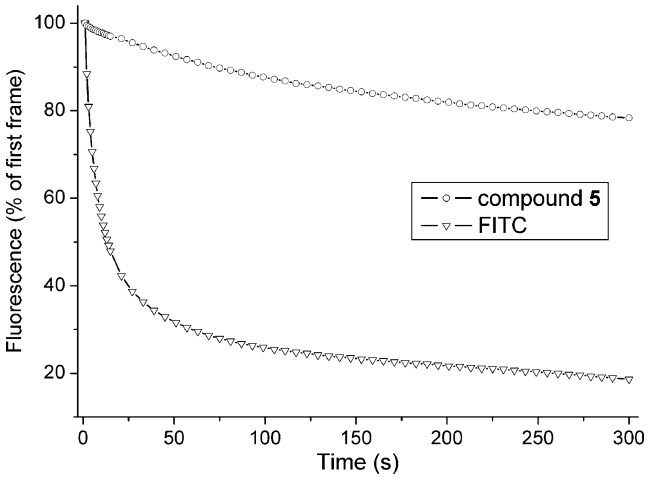

Fig. 5 Photobleaching kinetics of **5** and FITC. Data were collected by a CCD camera upon continuous and uniform illumination of liquid samples in a wide-field fluorescence microscope. The fluorescence was integrated across each frame and normalized for each sample to the initial signal

5 min, whereas the fluorescence of compound **5** has been reduced by only 20 % in same period of time. A 50% photobleaching lifetime around 1000 s was estimated for the new derivative, which is considerably longer than for typical fluoresceins. The increased photostability is in line with previous observations on 3-O-alkyl fluorescein ethers (11–14) and can be linked to the decrease in intersystem crossing probability caused by the alteration of D_{2h} symmetry of the xanthene ring.

The observed resistance to photobleaching recommends **5** for long-term observation of intracellular structures in confocal microscopy studies. Unique information about the heterogeneity and dynamics of biological systems can be learned from direct observations of individual biomolecules. However, single-molecule fluorescence experiments require exceptionally bright, stable and photo-resistant fluorophores. We tested the new fluorescein derivative in a custom-built confocal microscope that has single-molecule detection capability and enables the identification of single fluorophores by their spectrum or lifetime [6]. A confocal image of single molecules immobilized in a thin PMMA film on glass is shown in Fig. 6a. The variation of fluorescence intensity for a specific molecule, the intensity trajectory, and its fluorescence decay are shown in Fig. 6b–c. The fluorescence lifetime of **5** on the surface (ensemble average 3.45±0.25 ns) is higher than measured in solution (2.8±0.1 ns), but notably, the fluorescence lifetime of the biotinylated derivative **7** is the same in solution as when tethered to a BSA-biotin-streptavidin coated surface (~2.85 ns, data not shown). Constraints on the torsional motion of the xanthene ring may hinder a non-radiative decay pathway for the molecules embedded in polymer pores, giving a longer fluorescence lifetime and a higher fluorescence yield than the molecules in solution. Similar observations have been reported previously for cyanine dyes in viscous solutions [6, 21]. Figure 6b shows a typical single-molecule intensity trajectory of **5** excited continuously at 483 nm. On average, we detect 4.5×10^4 photons from the new dye prior to irreversible photobleaching (average of 750 different molecules), about 3 times more than the number of photons emitted by a FITC molecule under similar conditions (1.6×10^4, average of 300 molecules). This is a surprising result, considering that FITC has an approximately 10 times higher experimental sensitivity (extinction coefficient×quantum yield). However, this is in agreement with the bulk photostability measurements described above and it is very promising for future single-molecule biophysical studies using the synthesized molecule as a fluorescent label.

Conclusions

This work focuses on the synthesis and the comprehensive spectral characterization at both bulk and single-molecule level of a recently reported fluorescein derivative. The molecule is a bright fluorophore, exhibiting both high ab-

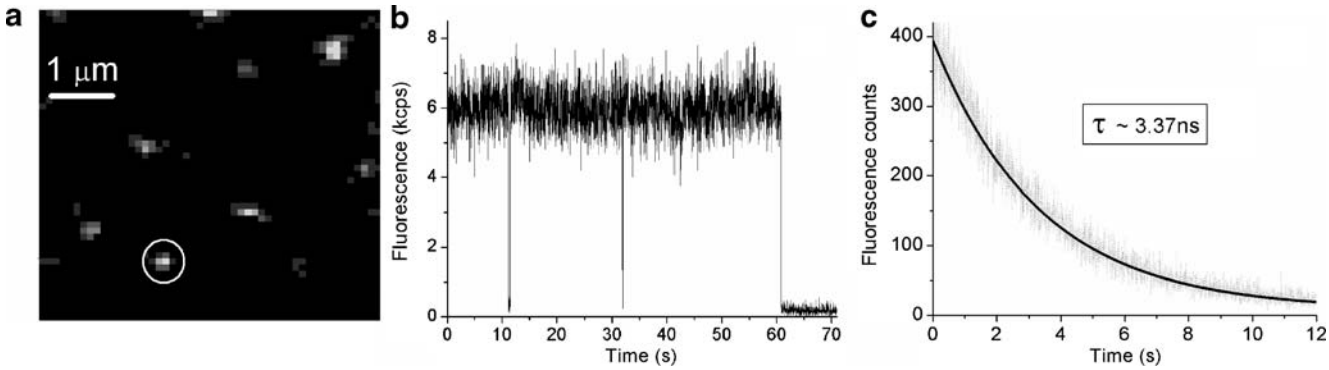

Fig. 6 **a** Confocal fluorescence image of single molecules of compound **5** embedded in a thin PMMA film on glass (5×5 μm, 100 nm and 5 ms per pixel, 100 W/cm^2). **b** The fluorescence intensity trajectory corresponding to the single molecule highlighted in (a). **c** The fluorescence decay of the molecule in (b) fitted to a mono-exponential function with a time constant of 3.37 ns

sorption coefficient and high fluorescence quantum yield. The absorption and emission spectrum, the fluorescence lifetime and the quantum yield display a remarkable pH invariance for a fluorescein dye. At least an order of magnitude increase in the photobleaching time for the new derivative was observed compared to FITC. The compound was extensively tested for single-molecule imaging and spectroscopy and it could be observed under continuous illumination for several seconds and minutes. In summary, our data show that fluorescein variant **5** is an excellent probe for long-term observation of intracellular structures and for future single-molecule experiments that require stable and long photo-resistant fluorophores. Significantly, the molecule has two readily accessible functional groups that can be activated or blocked independently. This feature makes it very versatile for fluorescence microscopy studies and it can serve, for instance, as bivalent inhibitor, a fluorescent bridge between two different recognition motifs.

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