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Title	Investigating tryptophan quenching of fluorescein fluorescence under protolytic equilibrium
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Publication Date	2009
Publication Information	Togashi, DM,Szczupak, B,Ryder, AG,Calvet, A,O'Loughlin, M (2009) 'Investigating Tryptophan Quenching of Fluorescein Fluorescence under Protolytic Equilibrium'. Journal Of Physical Chemistry A, 113 :2757-2767.
Link to publisher's version	http://dx.doi.org/10.1021/jp808121y
Item record	http://hdl.handle.net/10379/3984
DOI	http://dx.doi.org/http://dx.doi.org/10.1021/jp808121y

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Investigating Tryptophan Quenching of Fluorescein Fluorescence Under Protolytic Equilibrium.

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This is the author version, which may not contain all proofing corrections: The definitive version is the ACS published version which can be accessed at: DOI: [10.1021/jp808121y](https://doi.org/10.1021/jp808121y)

ABSTRACT

Fluorescein is one of most used fluorescent labels for characterising biological systems, such as proteins, and is used in fluorescence microscopy. However, if fluorescein is to be used for quantitative measurements involving proteins, then one must account for the fact that the fluorescence of fluorescein labelled protein can be affected by the presence of intrinsic amino acids residues, such as, tryptophan (Trp). There is a lack of quantitative information to explain in detail the specific processes that are involved and this makes it difficult to evaluate quantitatively the photophysics of fluorescein labelled proteins. To address this we have explored the fluorescence of fluorescein in buffered solutions, in different acid and basic conditions, and at varying concentrations of tryptophan derivatives, using steady-state absorption and fluorescence spectroscopy, combined with fluorescence lifetime measurements. Stern-Volmer analyses show the presence of static and dynamic quenching processes between fluorescein and tryptophan derivatives. Non-fluorescent complexes with low association constants ($5.0 - 24.1 \text{ M}^{-1}$) are observed at all pH values studied. At low pH values, however, an additional static quenching contribution by a sphere-of-action (SOA) mechanism was found. The possibility of a proton transfer mechanism being involved in the SOA static quenching, at low pH, is discussed based on the presence of the different fluorescein prototropic species. For the dynamic quenching process, the bimolecular rate constants obtained ($2.5-5.3 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$) were close to the Debye-Smoluchowski diffusion rate constants. In the encounter controlled reaction mechanism, a photoinduced electron transfer mechanism was applied using the reduction potentials and charges of the fluorophore and quencher, in addition to the ionic strength of the environment. The electron transfer rate constants ($2.3-6.7 \times 10^9 \text{ s}^{-1}$) and the electronic coupling values ($5.7-25.1 \text{ cm}^{-1}$) for fluorescein fluorescence quenching by tryptophan derivatives in the encounter complex were then obtained and analysed. This data will be applied to generate a more detailed, quantitative understanding of the photophysics of fluorescein when conjugated to proteins containing the amino acid tryptophan.

KEYWORDS: Fluorescein, Tryptophan, fluorescence quenching, electron transfer, bioconjugation.

1. Introduction

Widely applied in fluorescence imaging microscopy, the fluorophore labelled protein can be used to rapidly and easily visualise many different biochemical pathways, which involve protein interactions, protein expression, trafficking, intracellular signalling events, and cellular location.^{1,2} Many of the fluorophores used are designed to conjugate with specific amino acid residues or functional groups present in the target biomolecule. In many cases, the fluorophore is simply used as a contrast agent *i.e.* to show the location of the target biomolecule in a particular environment. However, for quantitative measurements of protein-surface interactions using techniques like Förster Resonance Energy Transfer (FRET), Fluorescence Lifetime Imaging (FLIM), or Fluorescence Correlation Spectroscopy (FCS)³⁻⁵ the possibility of changes in emission spectra, fluorescence intensity, or lifetime due to intramolecular or intermolecular factors can be significant, and adversely affect the interpretation of data. The photophysical parameters of a fluorophore are obviously dependant on various external environmental factors such as pH, polarity, temperature, ion concentration, membrane potential, etc.^{2,6,7} However, fluorophore emission after conjugation (covalent or otherwise) to a macromolecule can be completely different from the free probe, under the same environmental conditions,⁸⁻¹¹ due to the possibility that the emission properties may be affected by the microenvironment of the binding site, primarily, *via* non radiative mechanisms.¹²⁻¹⁵ Of particular significance is the quenching interaction of specific amino acids in the protein, like tryptophan.^{16,18}

Fluorescein and its derivatives are the most widely used family of fluorophores in biology.^{2,6,7} They are easily excited, reasonably photostable, and have high fluorescence quantum yields.^{2,6} In particular they are widely used in fluorescence microscopy and FRET studies.^{1,19} Unfortunately, fluorescein can exist in different species (prototropic forms) with dissimilar photophysical properties, depending on the environmental pH.²⁰⁻²²

Fluorescein in aqueous solution can exist as cationic (FH_3^+), neutral (FH_2), monoanionic (FH^-), and dianionic (F^{2-}) species (Scheme 1), the concentrations of which are determined by the pH.^{20,22} Furthermore, the neutral form can exist as three different isomers: quinoid (Q), zwitterion (Z), and lactone (L) forms.^{20a} Other environmental conditions such as ionic strength and temperature also have an impact on the equilibria.^{20b,23} The $\text{p}K_a$ values are normally determined from analysis of the electronic absorption spectra of fluorescein in an acid-base titration experiment. The direct determination of an accurate absorption spectrum for most of the individual prototropic species is unreliable, except for the dianion, because of the overlap between the individual spectral contributions in the absorption spectra. Therefore, to extract the individual absorption spectra for $\text{p}K_a$ measurements, one has to use a spectral resolution procedure, or a multi-species equilibrium model with the analysis of absorption changes at one or more wavelengths, or chemometric methods.^{20,22} Different $\text{p}K_a$ values have been reported because of the number of different approaches utilised. In general, the three $\text{p}K_a$ values are in the range 2.00-2.25, 4.23-4.4, and 6.31-6.7.^{20,-23} Recently, these values were corrected by using activity coefficients and reported as $\text{p}K_{a1} = 2.22$, $\text{p}K_{a2} = 4.34$, and $\text{p}K_{a3} = 6.68$.²³

Despite the fact that the majority of experimental observations reported in the literature are in agreement, there are some controversies related to the electronically excited states, specifically the identity of the excited state species, and the interconversion between excited state species.^{20,21} Under alkaline conditions ($\text{pH} > 8$), where the dianion is the dominant species in the ground state, the fluorescence spectral profile does not change with proton concentration. At acidic pH (~ 1.5 to ~ 5) where the dominant species of fluorescein in the ground state are cationic, neutral, and monoanion forms, the profile of the fluorescence spectra is also always the same. However, at near neutral pH (between ~ 5 to ~ 8) where the neutral, monoanion, and dianion are present, one observes changes in the profile of the fluorescence spectrum.

It is in the analysis and interpretation of these observations that the two main questions arise: (i) the contribution and importance of different neutral forms of fluorescein to the fluorescence emission,²⁰ and (ii) the effect of phosphate buffers on the excited state of the monoanion and dianion.^{20a-c,21} The first issue refers to the nature of the contribution to the measured fluorescence spectrum from the other species apart from the dianion, present at near neutral pH: this contribution is either composed of emission from both neutral and monoanion species (both species having very similar emission spectra),^{20a,d} or the emission originates only from the monoanion.^{20b,c} This controversy originates from the spectral analysis approach used in the different literature studies. One view is that irrespective of the exact structure of the neutral form, the neutral species is always non-fluorescent.^{20b,c} However, other studies, show that the neutral fluorescein species, in aqueous solution, exists as a combination of lactone, quinone, and zwitterion forms.^{20a} Of these neutral species, only the quinone form is fluorescent with a quantum yield of 0.29, which is similar in magnitude to the quantum yield of the monoanion (0.36).^{20a} In the same study, it was calculated that in the ground state, the non-fluorescent zwitterion species is present in the same concentration as the quinone.^{20a}

There is agreement in the literature, that at pH above ~ 1.5 that the fluorescence emission can be decomposed linearly into two spectra corresponding to the dianion and the non-dianion forms. A fast proton transfer equilibrium reaction occurs between pH ~ 1.5 to ~ 5 , which means that there is a fast interconversion between the cationic, neutral, and monoanion species, during the excited state lifetime. This conversion is estimated to be up to 85%.^{20b,c} In the pH region above ~ 5 , the conversion in the excited state between the non-dianion species to the dianion does not occur in water or in low phosphate buffer concentration. Therefore under the physiological conditions encountered in most bioscience applications, the dianion is the predominant fluorescein species present and it has a large absorption coefficient and high fluorescence quantum yield. It has been observed that fluorescein emission can be quenched by amino acids.¹⁶⁻¹⁸ A more detailed analysis of the amino acid quenching mechanism which takes into account the presence of other prototropic species, and the possibility for energy transfer quenching is still required for a comprehensive, quantitative understanding of fluorescein photophysics in proteins.

In this manuscript, we investigate in detail the fluorescence quenching of hydrolysed fluorescein diacetate (FDAH) by tryptophan derivatives (or indole core compounds) using UV-visible absorption and fluorescence emission and lifetime spectroscopy. The quenching process was studied under a pH range to ascertain the effect of the presence of different prototropic species. The Stern-Volmer constants and quenching rate constants were obtained. In addition, electron transfer parameters (ET) using diffusion Debye-Smoluchowski and ET Marcus models were applied to extract distance dependence data from the fluorescence quenching analysis. Our interest, in particular, is to obtain baseline photophysical data that can be applied to the detailed study of the photophysics of fluorescein conjugated to serum albumin proteins used for exacting quantitative fluorescence microscopy applications.

2. Experimental procedures

Materials: Fluorescein (F), Fluorescein Diacetate (FDA), Tryptamine (TrpA), and N-Acetyl-DL-tryptophan (AcTrp) were obtained from Sigma-Aldrich. The pH 2.0, 5.0, and 11.0 buffers with an estimated ionic strength of 0.07 M, 0.31 M, and 0.10 M, were obtained from FIXANAL®. The pH 7.4 buffer was made up using PBS tablets (Fluka), and had an ionic strength of 0.16 M.²⁴ All reagents were used as received without further purification. All aqueous solutions were made up with deionised water from a Milli-Q Millipore system.

Apparatus: Absorption spectra were recorded with a Perkin Elmer Lambda 950 UV-visible spectrophotometer in 2 mm path length quartz cell, with the sample held at room temperature

(21°C). Fluorescence spectra were made using a Cary Eclipse Fluorescence Spectrophotometer (Varian) and spectra were corrected by the correction curves provided by the manufacturer. Magic-angle fluorescence decays were recorded using a Time Correlated Single Photon Counting system (Fluotime 200, Picoquant GmbH, Berlin). The excitation at 440 nm was a pulsed laser diode (LDH-440, Picoquant GmbH) at 5 MHz, and the fluorescence detected at 520 nm. Typical full widths at half-maximum obtained for instrument response function are in the order of a hundred picoseconds, and were obtained using a diluted aqueous Ludox solution. All measurements were stopped at a count of 20,000 in the time channel of maximum intensity. Samples within μM concentration were held in a 1 mm path length quartz cuvette using front-surface excitation geometry to reduce as much as possible any inner-filter effects.

FDA hydrolysis: 5.6 mg FDA was dissolved in 2 ml methanol and then 1 ml of 1 M NaOH was added. The solution was then neutralised with approximately 83 μL of (37%) hydrochloric acid. The product of FDA hydrolysis is fluorescein at a final concentration of 2.9 mM, hereafter called FDAH.

Fluorescence quantum yields: Fluorescence quantum yields (Φ_f) were determined using Fluorescein in NaOH (0.1M), $\Phi_f = 0.72$,²⁵ as a standard, after applying necessary corrections for the refractive index of the medium. FDAH concentrations of $\sim 3 \mu\text{M}$ were used in all fluorescence measurements to keep the absorption below 0.06. The error in the estimation of Φ_f is $\pm 10\%$. All the fluorescence intensity measurements were carried out on non-deaerated samples at room temperature.²⁵

Quenching experiments: Because of solubility issues in buffered solutions, at pH 5.0, 7.4, and 11.0, stock solutions of 0.1 M AcTrp in 0.2 M NaOH were first prepared. Solutions of AcTrp at pH 5.0, 7.4, and 11.0 were generated by carefully monitoring, using a pH meter, the addition of small volumes of concentrated HCl. The final ionic strength of the stock solutions was ~ 0.2 M. Tryptamine hydrochloride (TrpA) 0.1 M stock solutions were prepared in the respective buffered solutions pH 2.0 and 5.0, and verified using a pH meter. For different quencher concentrations, a corresponding aliquot of a stock solution was taken and diluted with the buffer solution of respective pH. A final concentration of approximately 5 μM hydrolysed FDA was then used in all Stern-Volmer experiments.

Data analysis: Lifetime data were analysed using the FluoFit, Version 4.1 (PicoQuant) software package. The intensity averaged lifetime is calculated by $\tau_{av} = \sum f_i \tau_i$, where $f_i = a_i \tau_i / \sum a_i \tau_i$ is the contribution factor of the i^{th} exponential term, with a pre-exponential a_i and decay time τ_i . The goodness of the fit was assessed by having a chi-squared value of less than 1.2 and a residual trace that was symmetric about the zero axes. The lifetime errors which were typically less than 0.05 ns were calculated by using the error surface analysis provided by the software package with 99% probability on the chi-squared value.

3. RESULTS AND DISCUSSION

3.1. FDAH Prototropic species.

There were no significant differences in the steady-state absorption (Fig. 1A) or fluorescence (Fig. 1B) spectra of FDAH compared to the pure fluorescein standard recorded at different pH. This indicates that the FDA to FDAH hydrolysis process does not change the relative ion concentration for the fluorescein prototropic species. At pH 2.0 the absorption spectrum ($\lambda_{\text{max}} = 435$ nm) corresponds to the cationic form, however the presence of a shoulder near 480 nm indicates that some of the neutral form is also present, which is in agreement with reported studies.²⁰ Analysis of the prototropic equilibria $\text{p}K_a$ values ($\text{p}K_{a1} = 2.22$, $\text{p}K_{a2} = 4.34$, and $\text{p}K_{a3} = 6.68$),²³ indicates that there is no significant contribution from the anionic species to the ground state equilibrium at pH 2.0

(Table 1). However, the FDAH fluorescence spectrum obtained at pH 2.0, is very similar to the fluorescence spectrum of the anionic species. This is because of the fast cationic→neutral→anion equilibrium that is established in the excited state.^{20,21} Therefore, irrespective of whether the cationic or neutral form is excited, the species responsible for emission at pH 2.0 is the monoanion.

The main prototropic forms in the ground state equilibrium of FDAH at pH 5.0 and 7.4 are the monoanion (80%) and dianion (84%) respectively. Both forms contribute to the absorption spectra, e.g., the monoanion broadening the band at 490 nm (Fig. 1A) at pH 7.4. Both species also contribute to the fluorescence spectra, e.g. the dianion fluorescence overlaps the monoanion emission at pH 5.0 (Fig. 1B). The absorption and fluorescence spectra at pH 11.0 correspond almost entirely to the dianion.^{20,-22}

The existence of different prototropic species with a high degree of emission spectral overlap, and the presence or absence of excited state proton transfer equilibria, explain the large differences in the reported absolute fluorescence quantum yields for the different prototropic species ($\Phi_{FH_3^+}= 0, 0.39$ (0.9-1.0), $\Phi_{FH_2^-}= 0-0.30$, $\Phi_{FH^-}= 0.26-0.37$, and $\Phi_{F2^-}= 0.93$).^{20,-22}

3.2. Interaction between FDAH and tryptophan derivatives.

The UV-visible spectra of buffered solutions of FDAH are affected by the presence of tryptophan derivatives. In Fig. 2A (pH 2.0) and 2B (pH 5.0), there are absorption contributions from TrpA due to the high concentrations used. Conversely, the AcTrp absorption in alkaline media (Fig. 2C and 2D) is weaker than that observed for TrpA in acid media. Thus this extra contribution in the absorption spectra can mask significant FDAH interaction effects with tryptophan. Fortunately, this can be rectified by subtracting the absorption spectra of the corresponding pure solutions of the tryptophan derivatives at the same concentration, resulting to the FDAH absorption corrected spectra (Fig. 2A'-D').

The corrected FDAH absorption spectra show very small but consistent changes in the main absorption band, first a reduction in intensity, and second a red shift, with $\lambda_{max} = 436$ nm at pH 2.0, 452 nm at pH 5.0, 490 nm at pH 7.4, and 491 nm at pH 11. Furthermore, isobestic points were detected at approximately 448 nm, 485 nm, 498 nm, and 496 nm for pH 2.0, 5.0, 7.4, and 11.0, respectively. At pH 5.0 (Fig. 2B'), the small variations of absorption values at the maxima (< 0.002) made it very difficult to observe the isobestic point. However, increasing the FDAH concentration (2 fold) and pathlength (to 10 mm) enabled observation of the isobestic point. These changes in the absorption spectra are due to the formation of a weak association complex.

At pH 2.0 and 5.0, the charge of the main TrpA species is positive, because its $pK_a = 9.3$.^{26a} Assuming similar pK_a values for AcTrp and tryptophan ($pK_{a1}=2.38$, and $pK_{a2}=9.39$),^{26b} the charge of the main AcTrp species at pH 5.0 and 7.4 is zero, while at pH 11 AcTrp with -1 charge is dominant. If one considers the charges of the interacting species, then an electrostatic interaction may be responsible for formation of the weak complex observed in the absorption spectra. It is only at pH 5.0 where there are favourable conditions for complex formation because TrpA is positively charged and the main fluorescein form is negatively charged. In fact, the measured association constant for the complex reaches the highest value at pH 5.0 (*vide infra*). Under the other pH conditions studied, complex formation is much less likely because of unfavourable charges. However, the repulsive/attractive electrostatic interactions can be reduced in solutions of high ionic strength due to an ion screening process.²⁷

Fig. 3 shows the fluorescence spectra of FDAH in the presence of tryptophan derivatives. The fluorescence intensity maxima are found at 516 nm (554 nm, shoulder), 514 nm (553 nm, shoulder), 516 nm, and 516 nm for pH 2.0, 5.0, 7.4, and 11.0, respectively. In contrast to the absorption spectra and despite the decrease of intensity due to quenching, the fluorescence spectra do not show any changes in profile and are identical to those as shown in Fig. 1B. Furthermore, the unchanged

spectral shape of FDAH at all pHs studied is an indication that the presence of high concentrations of tryptophan derivatives does not affect the excited state equilibrium between the prototropic forms, that is, the presence of quencher does not affect the proton transfer process that may occur in the excited state.

3.3. FDAH fluorescence quantum yield and lifetime.

The fluorescence quantum yields and fluorescence lifetimes for FDAH obtained at different pH (Table 1) are similar to those obtained for the fluorescein standard under the same experimental conditions, indicating that the hydrolysis process did not affect the photophysical properties of the fluorescein moiety. All the fluorescence decays could be fitted to a single exponential function, which is expected if the contribution of one or two anionic species in the excited state is very small at the emission wavelength of 520 nm for the pH 2.0, 7.4, and 11.0. However, at pH 5.0 one would have expected a second lifetime component due to the presence of significant quantities of both monoanion and dianion FDAH prototropic forms as indicated by the emission spectrum (figure 1B).

If one assumes that the fluorescence emission at pH 11.0 originates only from the dianion and that the pH 2.0 fluorescence emission is due only to the monoanion, then the contribution of monoanion fluorescence to the fluorescence spectrum at pH 5.0 can be calculated using multi-linear regression of the pH 2.0 and 11.0 fluorescence spectra, obtained at the same concentration.²⁰ Figure 4 shows that a reasonable fit is obtained and one can ascribe the observed deviations to the fact that the spectra used were acquired under different buffers and ionic strength conditions. Using the ratio of the integrated areas, one can estimate the monoanion contribution to the fluorescence spectrum at pH 5.0 as being approximately 83%. The lifetime data is a little ambiguous because fitting of a bi-exponential model only shows a slight improvement relative to a mono-exponential model, and it was very difficult to reach a real solution when all the parameters are left free to adjust. When the fluorescence decay of fluorescein at pH 5.0 is fitted with a bi-exponential decay law function, using a fixed $\tau_1 = 4.00$ ns decay time, a second decay time, $\tau_2 = 3.37$ ns, with a 77 % intensity contribution is recovered. The two factors which may be responsible for the difficulty in obtaining an accurate bi-exponential fit are the small difference between the two decay times (τ_1 and τ_2) and the time resolution of the equipment.²⁸ The lifetime obtained using a single exponential fitting, 3.51 ns, is the same value as the average lifetime value from bi-exponential fit model. Therefore, for the analysis of the quenching experiments at pH 5.0, a single exponential model was used.

3.4. FDAH fluorescence quenching by tryptophan derivatives.

The Stern-Volmer plots using the quantum yield ratios of FDAH against TrpA and AcTrp concentration shows an upward curvature at all pHs studied (Figure 5) indicating a combination of dynamic and static quenching.²⁹ In the quenching experiments at pH 2.0 and 5.0 and with TrpA concentrations above 40 mM, the intensity decays are no longer well fitted by a single exponential function because of a fluorescence contribution of TrpA to the total emission. The fluorescence decays for buffered solutions of pure TrpA measured at 520 nm require a tri-exponential model (0.32 ns, 1.63 ns, and 5.23 ns for pH 2.0; 0.33 ns, 1.77 ns, and 4.98 ns for pH 5.0). Therefore, in the quenching experiments where the concentration of quencher was greater than 40 mM, the TrpA contributed a minor component (<15%) of the total fluorescence decay measured at 520 nm. When AcTrp was used as a quencher no extra contribution from the quencher fluorescence was observed in the fluorescence decays. In contrast to steady state results, the ratio values of lifetime with and without quencher (τ_0/τ) show a linear trend with TrpA and AcTrp concentrations (Figure 5). This is because of a dynamic quenching contribution to FDAH excited state quenching by TrpA and AcTrp. Furthermore, the presence of more than one fluorescent species does not affect the linearity of the dynamic quenching.

No evidence for protolytic equilibrium between the fluorescein monoanion and dianion in the excited state was found in our study. However, the excited state monoanion to dianion conversion has been observed in high concentration phosphate (0.02 M to 1 M) or acetate buffered solutions between pH 6 and 10.^{20c,21} In our case, none of buffers used for any of the measurements had phosphate anion concentration greater than 10 mM, significantly smaller than the concentration where the protolytic monoanion to dianion equilibrium was observed. If however, the protolytic equilibrium does occur, we assume (supported by the observation that the fluorescence spectral profiles do not change with the quencher concentration) that it takes place prior to the quenching process.

The decrease in fluorescence quantum yield caused by a combination of collisional, non-fluorescent complex formation, and a sphere-of-action (SOA) quenching mode can be described by the modified Stern–Volmer equation (Eq.1):

$$\left(\frac{\Phi_0}{\Phi}\right) \cdot \left(\frac{\tau_0}{\tau}\right)^{-1} = (1 + K_{ap}[Q]) \cdot \exp(V_m[Q]) \quad (1)$$

$\tau_0/\tau = 1 + K_{SV}[Q]$, where $K_{SV} = k_q \times \tau_0$ (k_q = bimolecular quenching constant, τ_0 = unquenched fluorescence lifetime, and $[Q]$ = quencher concentration). $K_{ap} = \varepsilon^* . K_C^{30}$ where K_C is the complex formation constant, ε^* the ratio of molar absorptivities of the complex and the fluorophore at the excitation wavelength, and V_m the molar volume of SOA with a radius:

$$R_{SOA} = \sqrt[3]{\frac{3000 \times V_m}{4 \times \pi \times N_a}} \quad (2)$$

where, N_a is Avogadro's number. The fitting results are shown in Figure 5 and Table 2.

3.4.1. Static quenching.

The non-fluorescent complexes of FDAH are weak as shown by the low values obtained for the apparent equilibrium constants. However, the degree of complex formation in solution is sufficient to cause the curvature observed in the Stern-Volmer plots. The curvature is more pronounced at pH 2.0 and 5.0 because two distinct static processes are present, complex formation and SOA. Marmé *et al.*¹⁶ have also observed a non-fluorescent complex between fluorescein and tryptophan at pH 7.4, which has an association constant value approximately double the value determined in the FDAH case. We believe that the differences observed are due to the different excitation wavelengths used because the apparent complex association constant depends on the absorption co-efficients which are wavelength dependant. The V_m values (Table 2) are found by fitting equation 1 to the Stern-Volmer experimental data (Figure 5A'-E'), while the R_{SOA} values are calculated using equation 2. The respective values for TrpA at pH 2.0 and 5.0, and for AcTrp at pH 5.0 are 15.6 Å, 13.2 Å, and 14.3 Å, while no SOA is obtained for pH 7.4 and 11.0. These R_{SOA} values are smaller than those obtained by Doose *et al.* (~19 Å), where the Oxazine MR121 fluorophore was used.¹⁸ The R_{SOA} values obtained are reasonable for an energy transfer quenching process, but this mechanism can be ruled out due to the very poor overlap between the donor fluorescence and acceptor absorption. The rate of electron transfer (k_{et}) estimated for a separation distance of ~14 Å is very low, with respect to the time scale of the static quenching process.

Doose *et al.* point out that probability of electron transfer at distances of ~19 Å was very low, however, they suggested that a potential long range attractive interaction between fluorophore and tryptophan (within ~ 20 Å) could influence the relative geometrical arrangement, enhancing quenching beyond the diffusion time scale, which would result in a SOA contribution to the static quenching process.^{17,18} Castanho and Prieto³¹ suggested that if the time resolution of the fluorescence lifetime measurement was ~ 0.5 ns, then the SOA can be interpreted as the sphere of radius R_s , within which the fluorophore can randomly move during that time resolution limit. This

means that non-exponential fluorescence decays^{29,31} that are commonly observed in transient effects in the diffusional quenching process can be simplified to a single exponential decay. R_s can be calculated by the simple expression:

$$R_s = \sqrt{\frac{5}{3}Dt} \quad (3)$$

where D is the mutual diffusion constant of the fluorophore and the quencher molecules and t the time interval for the random walk. If $D = 2 \times 10^{-9} \text{ m}^2\text{s}^{-1}$ ^{18,29,32} and $t = 0.5 \text{ ns}$,²⁸ then $R_s = 12.9 \text{ \AA}$. This shows that fluorescence quenching by diffusional collision that occurs within a sphere of radius 12.9 \AA can be considered to be instantaneous. Since $R_s \sim R_{SOA}$, the electron transfer mechanism can still be used to explain the instantaneous static fluorescence quenching in a bimolecular collisional process.

The curvature in the Stern-Volmer plots are small at pH 7.4 (Fig 5D) and 11.0 (Fig 5E) and as a consequence, a straight line fits the combined experimental data (Fig 5D' and 5E'). This indicates that one of the static quenching processes has a very small or negligible contribution to the overall static quenching. We propose that it is the sphere-of-action which can be eliminated since spectroscopic evidence shows complex formation at pH 7.4 and 11.0. However, we would also expect that the random walk based SOA quenching (within the equipment time resolution) should be present at pH 7.4 and 11.0. This inconsistency indicates that a different process operates under neutral or basic conditions compared to acidic environments.

Assuming that the fluorescence quantum yield is zero for the neutral form^{20b,22b,c} or that protonation may lead to the non-fluorescent zwitterionic^{20a} or lactone forms,³³ then protonation of the excited monoanion can be considered as a quenching process (see scheme 2). The TrpA species at pH 2.0 and 5.0, and AcTrp species at pH 5.0, are probably protonated, and so can transfer a proton to the monoanionic FDAH. The protonation of the monoanion to the neutral form can occur within the diffusional rate limit with values $\sim 5.4 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$.^{21b} If one considers the local tryptophan derivative concentration (V_m in table 1), the time required for a proton transfer within the R_{SOA} distance is less than 5 ps ($1/(5.4 \times 10^{10} V_m)$) approximately, or $k_{pt} > 2 \times 10^{11} \text{ s}^{-1}$, then, protonation of the monoanion can be considered as instantaneous within the instrument time resolution. The protonation can be even faster if hydrogen bonding networks in the solvation layers connecting the fluorophore and the quencher molecules are involved in the proton transfer.³⁴ If, however, the rate of the neutral species formation by protonation is not fast enough, then the reverse reaction to the excited monoanion can be competitive. In this case, a mechanism that involves both proton and electron transfer (Proton-Coupled Electron transfer),³⁵ cannot be ruled out. In any case, the same process is more difficult in alkaline media due to the lack of a labile proton from the quencher molecule. Further experiments using better time resolution instrumentation, changing viscosity or the kinetic isotopic effect can help determine if the proton transfer (or coupled with electron transfer) is relevant to the static contribution to the whole quenching process.

3.4.2. Dynamic quenching.

The k_q values show a clear decrease when the pH increases, and the value for pH 7.4 is in agreement with the bimolecular rate constant obtained by Marmé *et al.* using L-tryptophan as the fluorescein quencher.¹⁶ The bimolecular quenching process can be represented by the encounter controlled reaction mechanism³⁶ (scheme 3) and k_q can be calculated from:

$$k_q = \frac{k_{et}k_d}{k_{-d} + k_{et}} \quad (5)$$

where k_d is the diffusion rate constant, k_{-d} the dissociation rate constant for the diffusional encounter pair, and k_{et} is the electron transfer reaction rate constant. The k_d can be calculated using the Debye-Smoluchowski model for diffusion of ions^{27,37} (eq. 6):

$$k_d = \frac{2 \cdot N_a \cdot k_B T}{3000 \cdot \eta} \cdot \left(\frac{R_F}{R_T} + \frac{R_T}{R_F} + 2 \right) \cdot \frac{1}{R_{FT} \int_{R_{FT}}^{\infty} r^{-2} \exp[w(r, I)/k_B T] dr} \quad (6)$$

k_B is Boltzmann constant, T is the temperature, η the water viscosity (0.91 cP at 25°C), R_F and R_T are the hard sphere radii of the FDAH and the tryptophan derivatives, where $R_{FT} = R_F + R_T$. The $R_F = 4.4$ Å, and $R_T = 4.2$ Å for AcTrp, and $R_T = 3.8$ Å for TrpA.³⁸ I is the ionic strength of solution, and $w(r, I)$ is the work function for the charged reactants at the separation distance r in the presence of an ionic atmosphere based on ionic strength I calculated by eq. 7:

$$w(r, I) = \frac{z_F z_T \cdot e^2}{2 \cdot \epsilon \cdot r} \left[\frac{\exp(\beta \cdot \sigma_F)}{1 + \beta \cdot \sigma_F} + \frac{\exp(\beta \cdot \sigma_T)}{1 + \beta \cdot \sigma_T} \right] \exp(-\beta \cdot r) \quad (7)$$

where $\beta = \left(\frac{8\pi N_a e^2}{1000 \epsilon k_B T} \right)^{\frac{1}{2}}$, $z_F z_T$ is the ionic reactants charge product and ϵ is the static dielectric

constant of water at 25°C ($\epsilon=78.3$), e is the electron charge, σ_F and σ_T are the radii of the respective reagent molecule plus the radius of the dominant counter-ion in the ionic atmosphere. In this work, we assume that the contributions of the counter-ions to the total size of fluorophore and quencher molecules are small. Therefore, σ_F and σ_T are assumed to be R_F and R_T , respectively. The k_{-d} is calculated by using the Eigen treatment for the dissociation of ionic encounter pair (eq.8):

$$k_{-d} = \frac{k_B T}{2\pi \cdot \eta \cdot R_{FT}^2} \cdot \left(\frac{1}{R_F} + \frac{1}{R_T} \right) \cdot \frac{\exp[w(R_{FT}, I)/k_B T]}{R_{FT} \int_{R_{FT}}^{\infty} r^{-2} \exp[w(r, I)/k_B T] dr} \quad (8)$$

The diffusion parameters can be calculated by using a numerical integration (Simpson method) of equations 6 and 8 over the limit from R_{FT} to 5000 Å.

In the excited state, the dianion is dominant at pH 7.4 and 11.0, while at pH 2.0 and 5.0 the monoanion is the major species. For the quencher, charges were assumed to be single positive for TrpA at pH 2.0 and 5.0, zero for AcTrp at pH 5.0, and 7.4, and single negative at pH 11.0 for AcTrp. It is important to note that, the ionic strength of buffer solutions used in these quenching experiments was relatively high (>0.2 M). Therefore, when the additional ionic strength contribution due to the quenchers was taken into account in the k_d and k_{-d} calculations, a small variation in the k_d and k_{-d} values was obtained (Table 3). Furthermore, depending on the sign and magnitude of charges that are present in the fluorophore and quencher, the diffusion reaction can be accelerated or retarded. The electrostatic interaction also explains the hindered dissociation of the encounter pair in acid media, where the encounter pair is composed of species with opposite charges, while in alkaline media dissociation is enhanced by the repulsion between the ions of same charge.

The k_{et} values can be recovered from k_q by using equation 5. The k_{et} values are most likely due to a photoinduced electron transfer between tryptophan acting as an electron donor, to the fluorescein molecule. We can assess the k_{et} value by using the rate constant electron-transfer reactions from semi-classic and non-adiabatic description from Marcus theory.³⁹

$$k_{et} = \frac{4\pi^2 H_{FT}^2}{h(4\pi\lambda k_B T)^{\frac{1}{2}}} \exp \left[-\frac{(\lambda + \Delta G^0)^2}{4\lambda k_B T} \right] \quad (9)$$

where h is Planck's constant, H_{FT} is the electronic coupling coefficient related to vibration, distance, and orientation of the reacting species, and λ , the reorganization energy, which has motion

contributions from the atoms of the reactants and the solvent reorganization free energy in an ionic atmosphere situation. ΔG^0 is the driving force of the reaction⁴⁰ determined by the redox potentials of the FDAH, $E^0(A/A^{\bullet-})$, in the excited state at vibrational zero electronic level (additional $\Delta E_{0,0}$ energy), and the tryptophan derivatives, $E^0(D^{\bullet+}/D)$, plus the work terms $w(D^{\bullet+}A^{\bullet-}) - w(DA)$, that is:

$$\Delta G^0 = E^0(D^{\bullet+}/D) - E^0(A/A^{\bullet-}) + w(D^{\bullet+}A^{\bullet-}) - w(DA) - \Delta E_{0,0} \quad (10)$$

The Coulombic interaction experienced by the reactants and products as they are brought together in the encounter pair are included in equation 10 as being $w(DA)$ and $w(D^{\bullet+}A^{\bullet-})$, respectively, and calculated using equation 7.

The redox potential used in the k_{et} calculation can be obtained from electrochemical data accessible in the literature. Using results from Tommos *et al.*⁴¹ the $E^0[\text{Trp}^{\bullet+}/\text{Trp}]$ vs. NHE are 1.07 V, 0.99V, 0.88V, and 0.64 V at pH 2.0, 5.0, 7.4, and 11.0, respectively. In the case of the fluorescein dianion the reduction potential $E^0[\text{F}^{2-}/\text{F}^{3\bullet-}] = 0.91$ V vs. NHE⁴² was used. The monoanionic reduction potential, $E^0[\text{FH}^-/\text{FH}^{2\bullet-}]$, can be estimated following Compton *et al.*'s suggestion⁴³ to use the $\text{p}K_a$ values of FH^- to $\text{FH}^{2\bullet-}$ (6.68²³ and 9.5⁴⁴, respectively), and the reduction potential of F^{2-} to $\text{F}^{3\bullet-}$. Applying the Nernst general equation for an equilibrium situation, $E^0[\text{FH}^-/\text{FH}^{2\bullet-}] = 0.74$ V vs. NHE is found. Finally, the total reduction potential for the electron acceptor centre has to be added to the excited single state energy (E_S) of 2.40 eV for both prototropic forms (monoanionic and dianionic), because both have approximately the same fluorescence maximum ~ 515 nm observed in all the buffer conditions used. The electron transfer driven forces for diffusional encounter pairs: $\text{FDAH}^{-1}/\text{TrpA}^{+1}$ (pH 2.0), $\text{FDAH}^{-1}/\text{TrpA}^{+1}$ (pH 5.0), $\text{FDAH}^{-1}/\text{AcTrp}^0$ (pH 5.0), $\text{FDAH}^{-2}/\text{AcTrp}^0$ (pH 7.4), and $\text{FDAH}^{-2}/\text{AcTrp}^{-1}$ (pH 11) using the above values are shown in the table 3.

Götz *et al.*⁴⁵ applied femtosecond absorption spectroscopy to show that fluorescein is electron photoreduced by either tryptophan or tyrosine after binding to Anticalin, a Lipocalin protein, where the fluorescein trianion radical is formed very quickly in about 400 fs. The ΔG^0 value used by Götz *et al.* is in the same range as found in our work.

The solvent reorganization energy, λ_s , can be calculated (equation 11) using the dielectric continuum model of Marcus,⁴⁶ where n is the refraction index of the solvent and the rest of the parameters having been defined previously:

$$\lambda_s = \frac{e^2}{4\pi\epsilon_0} \left(\frac{1}{2R_F} + \frac{1}{2R_T} - \frac{1}{R} \right) \left(\frac{1}{n^2} - \frac{1}{\epsilon} \right) \quad (11)$$

Electron transfer may occur when the molecules are close to each other, and then the dynamics of reaction are strongly dependent on the separation distance. For a contact distance of $R = R_{FT}$, then $\lambda_s \sim 0.96$ eV for TrpA and ~ 0.92 eV for AcTrp. The internal reorganization energy used by our calculation is the same one estimated by Götz *et al.*⁴⁵, which is equal to 0.42 eV. Therefore, the total reorganization energy λ , which is equal to the solvent reorganization energy plus the internal reorganization energy, is 1.34 eV for AcTrp and 1.40 eV for TrpA.

Combining the electron transfer equation 9 and the diffusional rate equations (equation 6 and 8), with equation 5, and using the reasonable parameters mentioned above, the electronic coupling H_{ab} can be calculated from the experimental value k_q . As noted in Table 3 the driving force increases ($-\Delta G^0$) in the opposite direction to that of the electron transfer rate, as a result of decreasing electronic coupling between the ionic species. This indicates that a spatial reconfiguration between the reaction centres may be necessary to promote more efficient electron transfer; however, the electrostatic interaction between the quencher and fluorophore may prevent the system from

reaching this ideal geometrical configuration. For example, in an investigation of photoinduced electron transfer in fluorescein, Miura *et al.* found a very small coupling (7.0 cm^{-1}) despite both quencher and fluorophore being covalently bound.⁴⁷ They suggest that the carboxylic group prevents free rotation between the donor and acceptor centres giving the centres an orthogonal orientation. In the case of charged FDAH and tryptophan derivatives, we propose that molecular repulsion may prevent the necessary geometrical alignment required to provide better coupling. However, if the electrostatic interaction is attractive, it would provide a better alignment of the donor and acceptor centres, and thus improving the coupling. For comparison, the values for the electronic coupling obtained here are around 20 fold smaller than the electronic coupling between the fluorescein and tryptophan in the Anticalin protein.⁴⁵ In the Anticalin protein, the electron transfer is assumed to be barrierless and therefore the reorganization energy is taken to be the same value as that of the driving force. Nevertheless, in the Anticalin case, fluorescein is located in the pocket where the geometrical arrangement is appropriate for a very efficient electron transfer process. Therefore, the electronic coupling in the Anticalin case reaches a maximum value at the closest distance between the reaction centres. To validate the electronic coupling of FDAH and tryptophan in the diffusional encounter pair (Table 3), we can apply the distance dependant electronic coupling under the same assumptions applied to the Anticalin case by using eq. 12.

$$H_{FT}^2 = H_{FT,0}^2 \exp[-\beta(R - R_0)] \quad (12)$$

where $H_{FT,0}$ is the electronic coupling matrix element for a donor-acceptor pair at van der Waals separation R_0 , and β is a decay constant scaling the electronic coupling and R the encounter distance between quencher and fluorophore. If we take typical values of 3.5 \AA for a co-planar distance between fluorescein and tryptophan centres,⁴⁵ $1-1.65 \text{ \AA}$ as the β value,^{39,45} 170 cm^{-1} for the $H_{FT,0}$ coupling found in the Anticalin case,⁴⁵ then the H_{FT} coupling is in the range $2.5-16.2 \text{ cm}^{-1}$ for AcTrp and TrpA when the same $R = R_{FT}$ distance is used for the above calculations and after applying equation 12. In other words, the coupling in the encounter complex is similar to that observed in Anticalin, with respect to the electronic coupling distance dependence.

4. CONCLUSIONS

Fluorescein is a very widely used fluorescent label in biological science however the factors that may affect the intensity of its fluorescence when bioconjugated are not fully understood. In this work, we have shown that the fluorescence of fluorescein, at different pH, in the presence of tryptophan is a very complex process. Absorption spectroscopy data and Stern-Volmer analyses show the presence of non-fluorescent, fluorescein-tryptophan complexes at all pHs studied. In the static quenching process, sphere-of-action (SOA) is also clearly present in acidic media (pH 2.0 and 5.0), while in alkaline media, SOA is not observed at the range of tryptophan concentrations (<70 mM) used in this study. We surmise that the difference in mechanisms is due to proton transfer from the quencher molecules that enhance the SOA in acid media. In the dynamic quenching process, electron transfer parameters were determined at all the pH studied. The electronic coupling between the main prototropic species and the indole based molecules TrpA and AcTrp show some dependence on the net charge involved in the formation of diffusion paired complexes, which can dictate the spatial organization between the reaction centres for electron transfer. Therefore, changes in fluorescence intensity and lifetime of the fluorescein in a labelled protein should not be directly correlated only to the external factors when amino acids such as tryptophan are located close to the conjugated probe.

ACKNOWLEDGMENTS

This work was supported by funding from a Science Foundation Ireland Principal Investigator Grant (number 02/IN.1/M231 to AGR) and the National Biophotonics Imaging Platform, an Irish Higher Education Authority Programme for Research in Third Level Institutions supported project.

TABLE 1 – Percentage contribution of fluorescein prototropic species, and photophysical parameters for FDAH and Fluorescein (in brackets) under different experimental conditions.

pH	C, N, M, D (%) ^{a)}	Φ_f	τ_f /ns
2.0	68, 32, 0, 0	0.20 (0.19)	2.91 (2.91)
5.0	0, 18, 80, 2	0.23 (0.25)	3.53 (3.51 ^{b)})
7.4	0, 0, 16, 84	0.58 (0.59)	4.01 (3.97)
11.0	0, 0, 0, 100	0.63 (0.64)	4.04 (3.97)

a) C (cation), N (neutral), M (anion), and D (dianion), b) as biexponential: 4.00 ns (fixed) and 3.37 ns with fractional intensity of 23% and 77%, respectively.

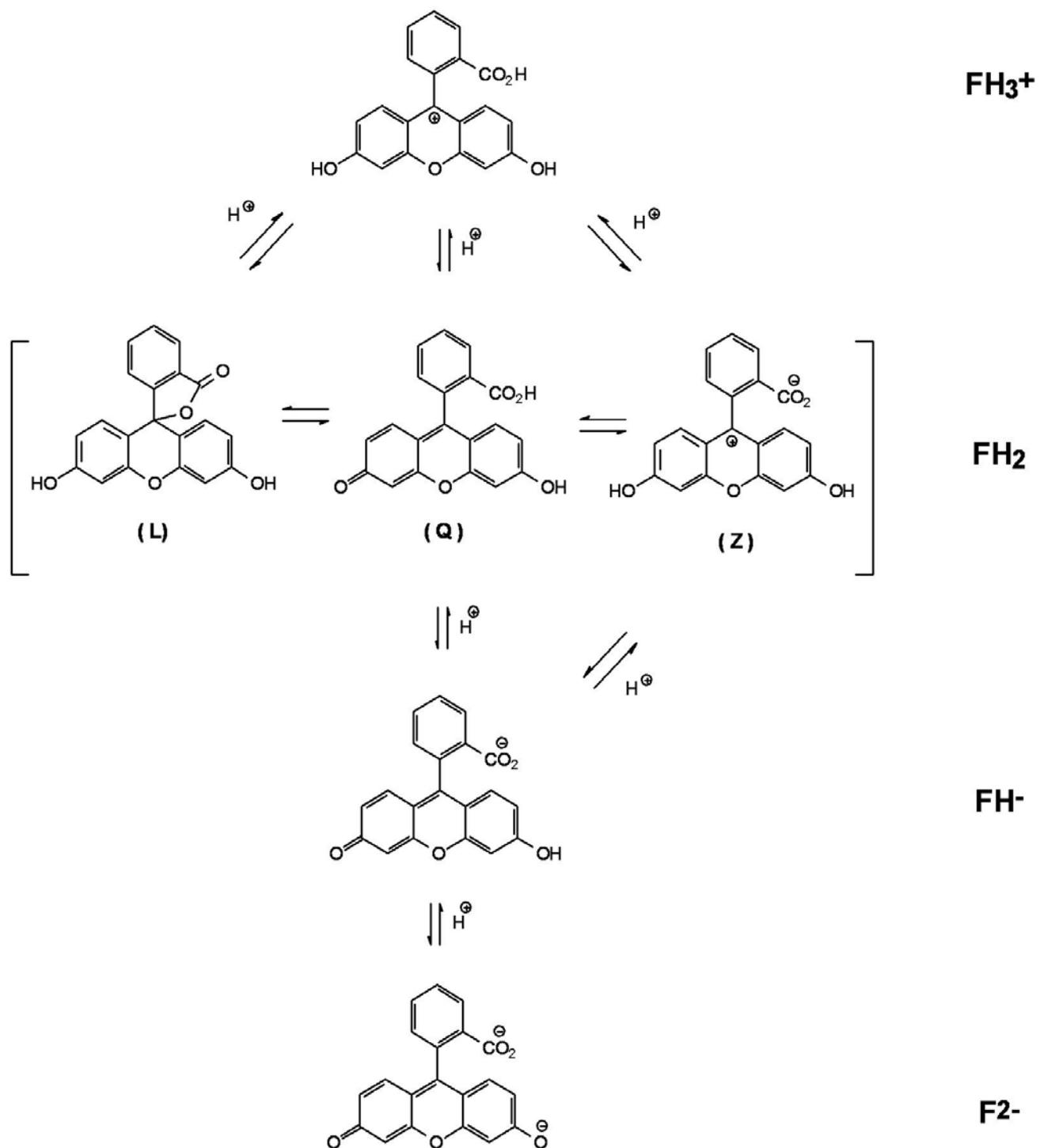
TABLE 2 – Quenching parameters for Stern-Volmer plots.

Quencher	pH	τ_0 / ns	K_{sv} / M ⁻¹	$k_q/10^9$ M ⁻¹ s ⁻¹	K_{ap} / M ⁻¹	V_m / M ⁻¹
TrpA	2.0	2.91	15.3	5.29	3.1	9.6
TrpA	5.0	3.51	15.8	4.50	12.6	5.9
AcTrp	5.0	3.53	12.2	3.46	2.7	7.3
AcTrp	7.4	4.01	10.0	2.52	6.7	—
AcTrp	11.0	4.04	9.9	2.45	5.0	—

TABLE 3 – Diffusion rate constant, diffusion dissociation rate constants, and electron transfer rate constants extracted from FDAH fluorescence dynamic quenching parameters.

pH	$Z_F.Z_T$	$k_d / 10^9$ M ⁻¹ s ⁻¹	$k_{-d} / 10^9$ s ⁻¹	$k_{el} / 10^9$ s ⁻¹	$-\Delta G^0$ / eV	H_{FT} /cm ⁻¹
2.0	(-1).(+1)	8.48-8.18	3.92-4.12	6.23-6.67	0.59	25.1
5.0	(-1).(+1)	7.83-7.77	4.45-4.52	6.19-6.07	0.67	17.0
5.0 ^{a)}	(-1).(0)	7.36	4.59	4.08-3.99	0.67	11.6
7.4	(-2).(0)	7.36	4.59	2.45-2.30	0.62	9.6
11.0	(-2).(-1)	5.78-6.36	7.27-6.53	4.72-4.53	0.86	5.7

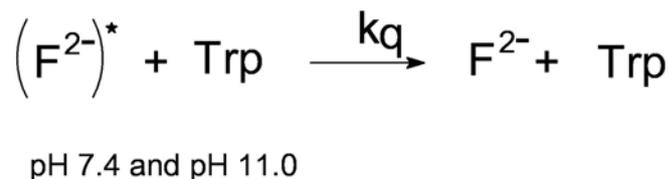
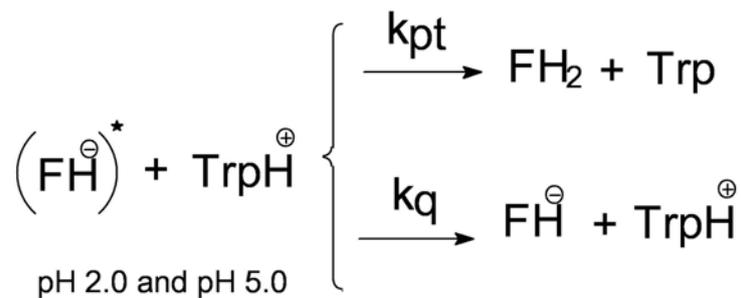
a) AcTrp used as the quencher.



SCHEME 1 – Ground State fluorescein protolytic equilibria.



SCHEME 2 – Bimolecular fluorescence quenching mechanisms.



SCHEME 3 – Quenching mechanism for excited fluorescein monoanion and dianion prototropic forms.

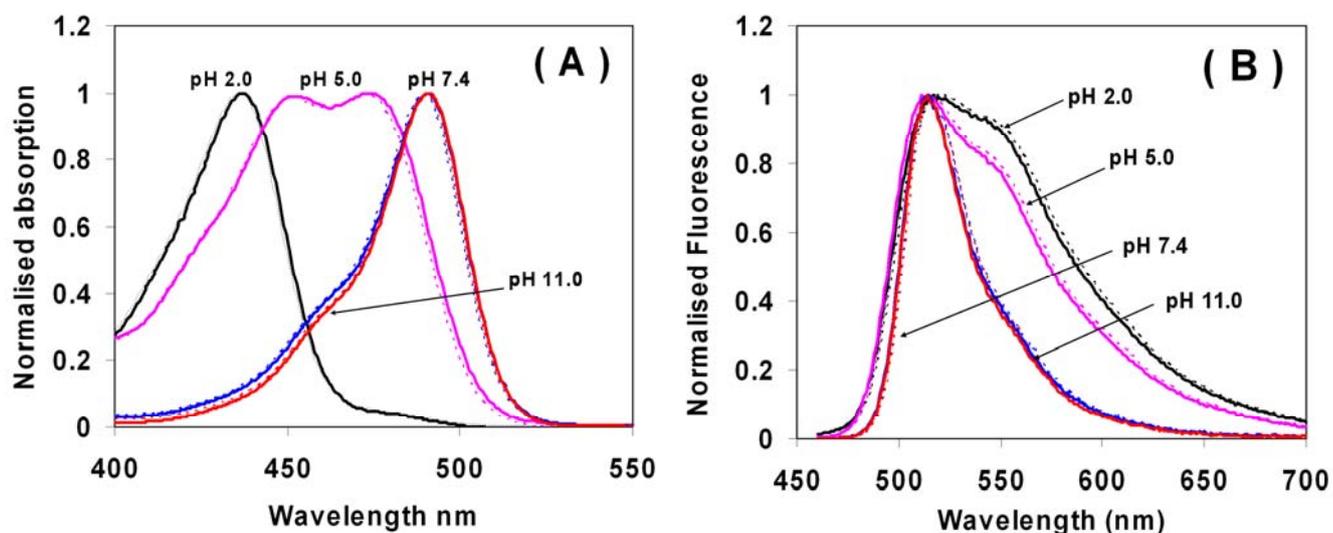


Figure 1 – Normalised absorption (A) and fluorescence (B) spectra for FDAH (solid lines) and Fluorescein (dashed lines) at different pH and in buffered solutions. Note that the fluorescein absorption and fluorescence spectra have been offset by 1 nm on the wavelength scale for clarity. Otherwise all spectra would perfectly overlap the corresponding absorption and fluorescence spectra of FDAH.

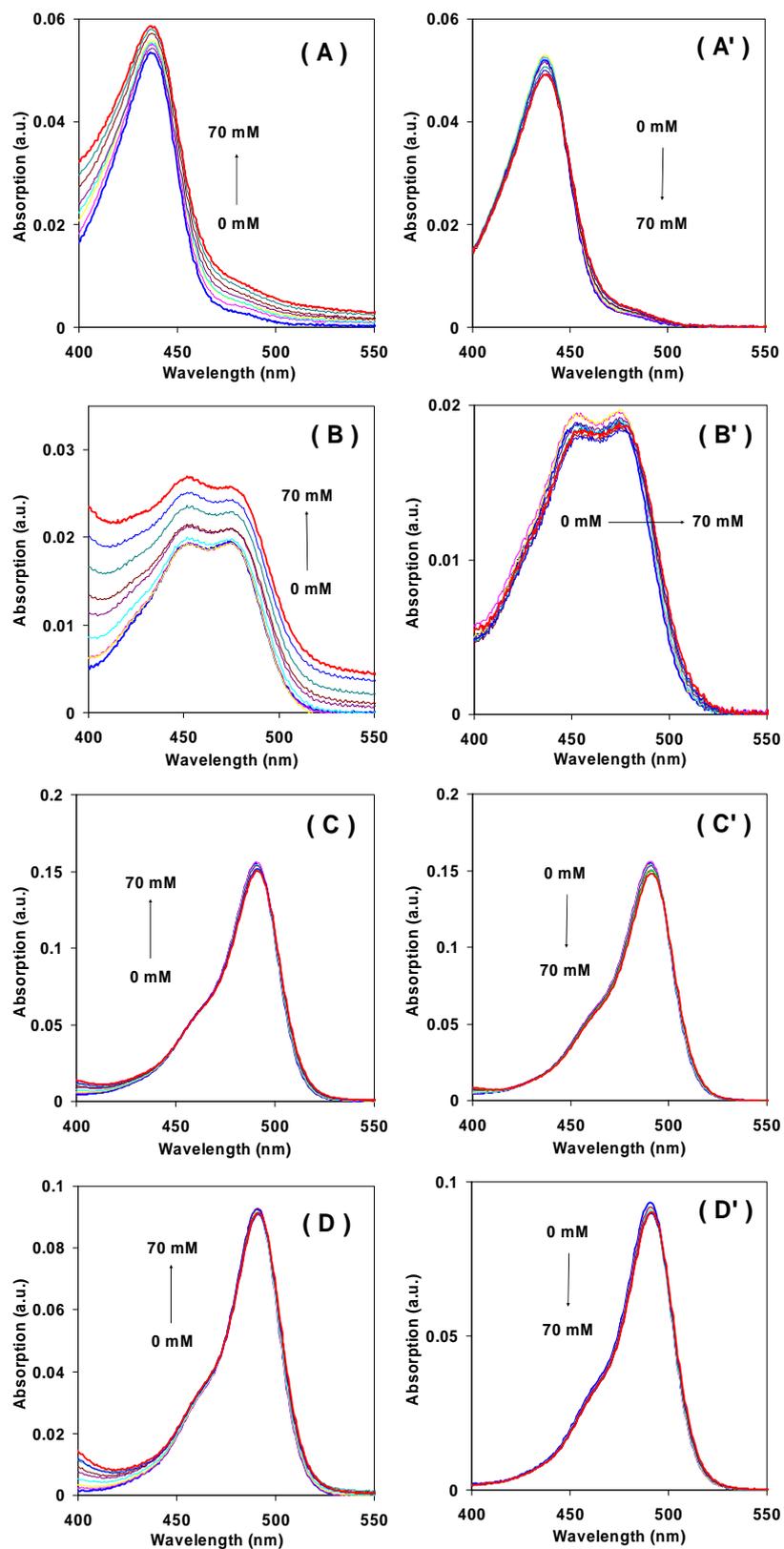


Figure 2 – Absorption spectra for FDAH with various concentrations of TrpA and AcTrp in buffered solutions at pH 2.0 (A, A'), pH 5.0 (B, B'), pH 7.4 (C, C'), and pH 11.0 (D, D'). The prime superscript denotes the corresponding corrected absorption spectra (see text).

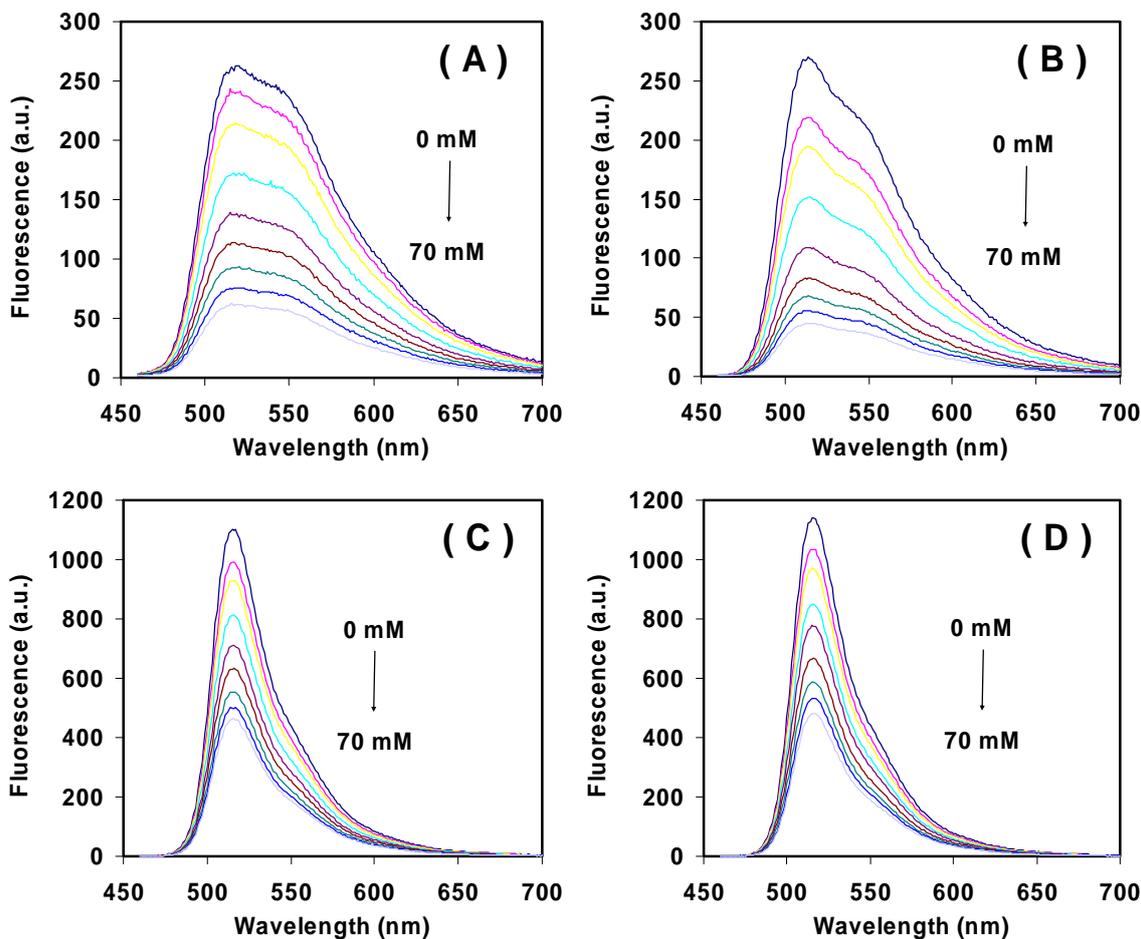


Figure 3 – Fluorescence spectra for FDAH with varying TrpA and AcTrp concentrations at pH 2.0 (A), pH 5.0 (B), pH 7.4 (C), and pH 11.0 (D) in buffered solutions.

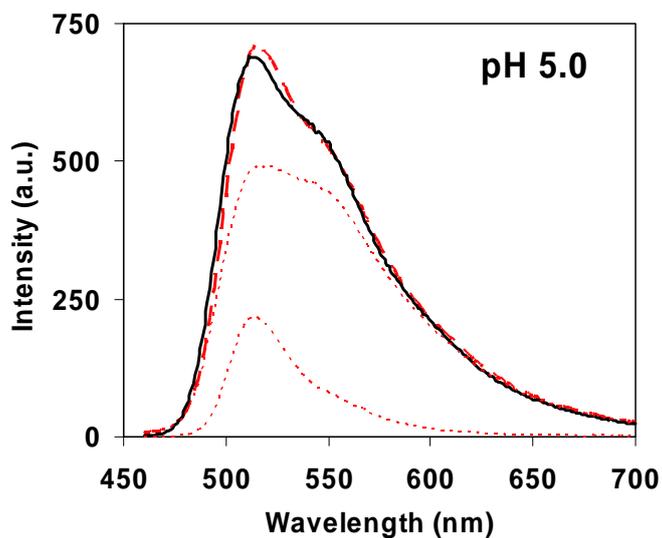


Figure 4 – Fluorescence spectra of fluorescein using 450 nm excitation (solid line) at pH 5.0 and recovered fluorescence spectra (dashed line) by multi-linear correlation of fluorescence spectra of fluorescein in pH 2.0 and pH 11.0 solutions. The dotted line spectra show the relative contributions of the pH 2.0 and pH 11.0 fluorescence spectra of fluorescein to the total fluorescence spectrum at pH 5.0.

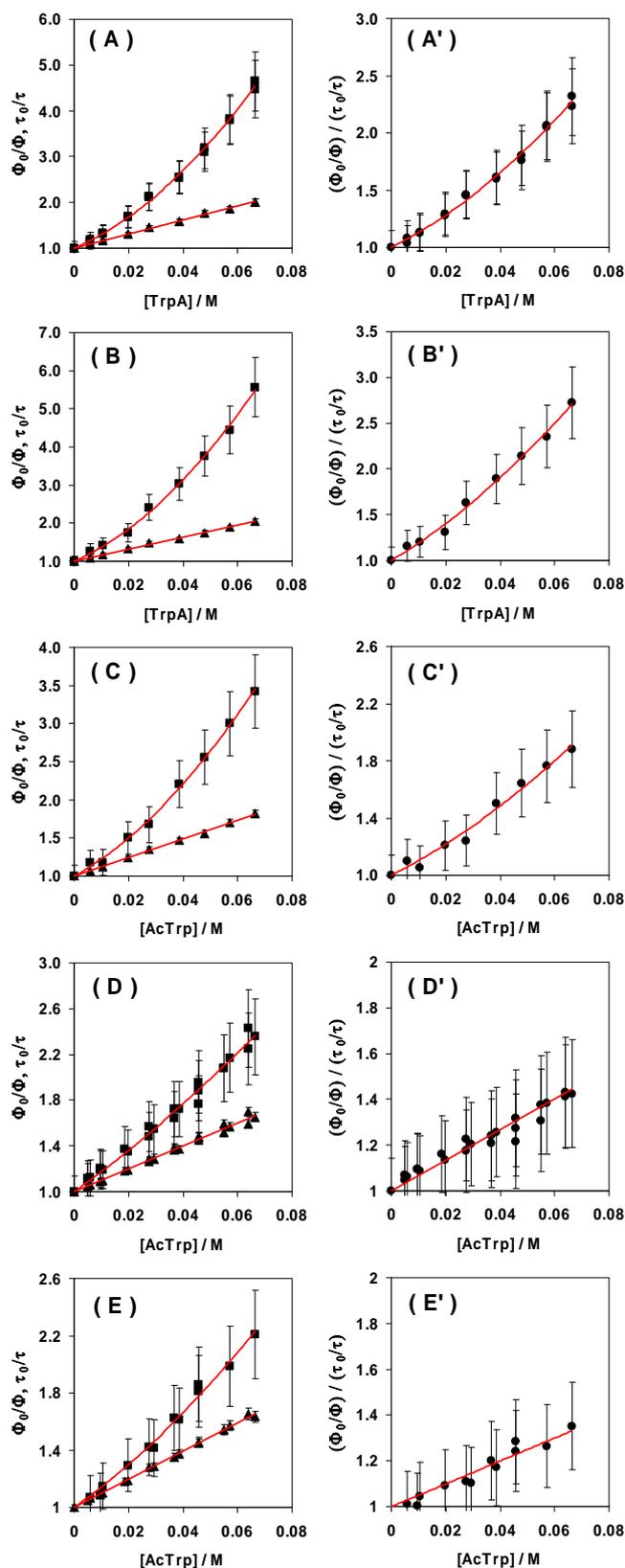


Figure 5 – Stern-Volmer plots for FDAH fluorescence quenching by TrpA and AcTrp in buffered solutions at pH 2.0 (A, A'), pH 5.0 (B, B'), pH 7.4 (D, D'), and pH 11.0 (E, E'). The prime superscript denotes the corresponding Stern-Volmer plots for the ratio between Φ_0/Φ and τ_0/τ . The solid square and triangle symbols represent the steady state and the time resolved experimental data. All the solid lines are from the equation 1 fitting model (see text). The error bars are calculated using the propagation of errors method for each one of experimental values.⁴⁸

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