Methods and Applications in Fluorescence

Determining a fluorophore’s transition dipole moment from fluorescence lifetime measurements in solvents of varying refractive index

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Abstract
The transition dipole moment of organic dyes PM546 and rhodamine 123 is calculated from fluorescence lifetime measurements in solutions of different refractive index. A model proposed by Toptygin et al (2002 J. Phys. Chem. B 106 3724–34) provides a relationship between the radiative rate constant and refractive index of the solvent, and allows the electronic transition dipole moments to be found: it is (7.1 ± 1.1) D for PM546 which matches that found in the literature, and (8.1 ± 0.1) D for rhodamine 123. Toptygin’s model goes further in predicting the shape of the fluorescent dye and here we predict the shape of PM546 and rhodamine 123 to be ellipsoidal.

1. Introduction

1.1. The fluorescence lifetime is a function of the refractive index of its environment
Fluorescence lifetime measurements have been used to probe a fluorophore’s environment for some time [1]. The fluorescence lifetime $\tau$ is defined as the average time a fluorophore remains in the excited state after excitation, and is the inverse of the sum of the radiative rate constant $k_r$ and the non-radiative rate constants $k_{nr}$ for the excited state depopulation processes

$$\tau = \frac{1}{k_r + k_{nr}} \tag{1}$$

Fluorescence lifetime measurements have been employed to sense the pH [2], viscosity, temperature, oxygen [3], glucose and ion concentrations, interaction with other molecules and, particularly relevant for this study, the refractive index. A key feature of the fluorescence lifetime is that it is independent of the fluorophore concentration, which is important for fluorescence lifetime imaging (FLIM) microscopy of cells and tissues where the fluorophore concentration is difficult to control. FLIM has, for example, been used to monitor apoptotic cells to study anti-cancer drugs [4] and in many other studies [5–8].

The reason why the fluorescence lifetime is a function of the refractive index $n$ is because $k_r$ is a function of $n$ [9]. One definition of the refractive index is the ratio of the speed of light in vacuum divided by the speed of light in the medium, and it varies with the type of solvent and the pressure or temperature of the solvent. It has been predicted theoretically that the fluorescence lifetime depends on the refractive index, and it has also been demonstrated experimentally by measuring fluorescence decays in solvents or solvent compositions of different refractive index, or varying the solvent refractive index with pressure, including supersonic jet-spectroscopy in vacuum [10]. Fluorescent dyes, fluorescent proteins CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) [11, 12] as well as GFP (Green Fluorescent Protein) [10, 13–15], lanthanides [16], quantum dots [17] and nanodiamonds [18] have all been shown to have a refractive index-dependent fluorescence lifetime. This effect has been used to study GFP infiltration into the nanochannels of mesoporous silica particles [19], and in supramolecular decay engineering, where complexion of rhodamine 6G by CB7 (cucurbit[7]uril) has been reported to increase the rhodamine 6G lifetime due to the low refractive index which a rhodamine 6G guest molecule experiences inside the CB7 cavity [20]. Probe penetration into lipid bilayers has also been assessed by lifetime measurements of various dyes, due to the high lipid refractive index affecting the fluorescence decay [21, 22].
In the particular case of GFP, the non-radiative rate constant seems to be insensitive of the environment, as the GFP fluorophore is tightly bound inside its barrel, protected from solvent effects, oxygen quenching and other diffusion-controlled collisional quenching processes—influences fluorescent dyes in solution are generally subjected to. The range over which the GFP decay senses the refractive index can be large, in the order of the wavelength of the light, depending on the experimental details [14]. It plays a role in total internal reflection FLIM, since GFP in close proximity to a glass-water interface has a lower average decay time than far away from the interface [14], and has been used to explain the shortened lifetime of GFP-labelled proteins in fixed cells in certain mounting media compared to living cells [23, 24].

In addition, this effect has been exploited to show that GFP-tagged proteins have a faster decay in the cell membrane compared to the cytoplasm, owing to the membrane’s higher refractive index [25]. In another fluorescence microscopy study, the fluorescence decays of cytoplasmic GFP and tdTomato, a red fluorescent protein, were mapped during mitosis, showing that the average GFP and tdTomato lifetimes remained constant during mitosis but rapidly shortened at the final stage of cell division [26]. The interpretation of this observation put forward was that the concentration of proteins— which have a high refractive index—in the cell changes during the cell cycle. Moreover, using flow cytometry and the GFP fluorescence lifetime of the cytoplasmic parameter, it has been shown that the GFP fluorescence lifetime can be correlated to changes in the subcellular localization of GFP-LC3 (microtubule-associated protein light chain 3) fusion protein to the autophagosome during autophagy [27].

1.2. Theory

The radiative rate constant \(k_r\) is related to the absorption and fluorescence spectra, and is a function of the refractive index of the medium surrounding the fluorophore according to

\[
k_r = 2.88 \times 10^{-9} n^2 \frac{\int F(\nu) d\nu}{\int F(\nu) \nu^3 d\nu} \int \frac{\varepsilon(\nu)}{\nu} d\nu \tag{2}
\]

where \(n\) is the refractive index, \(F\) is the fluorescence emission, \(\varepsilon\) the extinction coefficient and \(\nu\) the wavenumber (\(\nu = \lambda^{-1}\), \(\lambda\) wavelength). Equation (2) is known as the Strickler–Berg equation [28]. Hirayama and Phillips suggested that if the extinction coefficient \(\varepsilon\) did not vary with the refractive index, then \(k_r\) would be directly proportional to \(n^2\) [29]. Later Hirayama et al experimentally supported the \(n^2\) dependence [30].

\(F\) is the emission intensity and is related to the characteristic wavenumber \(v_{em}\) of the emission spectrum by

\[
v_{em}^2 = \frac{\int F(\nu) d\nu}{\int F(\nu) \nu^2 d\nu} \tag{3}
\]

The transition dipole moment \(M_{01}\) is associated with the transition from the ground state (0) to the excited state (1) due to charge displacement. This is related to the extinction coefficient via the following equation [31, 32]:

\[
M_{01} = 9.58 \times 10^{-2} \left( \frac{(2n^2 + 1)^2}{9n^4} \int \frac{\varepsilon(\nu)}{\nu} d\nu \right)^{1/2} \tag{4}
\]

where \(\nu\) is expressed in \(\text{cm}^{-1}\). Therefore by rearrangement of equations (3) and (4) and assuming that the electronic transition moment for absorption \(M_{01}\) is the same as it is for emission, \(M_{10} = (M_{01} = M)\) can be expressed as a function of \(k_c\):

\[
M = 1.786 \times 10^9 \left( \frac{(2n^2 + 1)^2}{9n^4} \frac{k_c}{v_{em}^2} \right)^{1/2} \tag{5}
\]

Recently however, in the context of tryptophan fluorescence, Toptygin et al have proposed an advanced model where the relationship between the radiative rate constant and the refractive index of the solution can not only yield the electronic transition dipole but the shape of the fluorescent molecule [33]:

\[
k_r \frac{v_{em}^2}{\nu} = \frac{\gamma n_0^5 \cos^2 \theta_x}{[n_0^2 + (n_1^2 - n_0^2)L_x]^2} + \frac{\gamma n_0^5 \cos^2 \theta_y}{[n_0^2 + (n_1^2 - n_0^2)L_y]^2} + \frac{\gamma n_0^5 \cos^2 \theta_z}{[n_0^2 + (n_1^2 - n_0^2)L_z]^2} \tag{6}
\]

where \(n_0\) is the refractive index of the solution and \(n_1\) is the refractive index of the particle. \(L_x, L_y, L_z\) are the depolarization factors which sum to unity and cannot be negative and \(\theta_x, \theta_y, \theta_z\) are the angles of the dipole moment in the ellipsoid axes, \(x, y,\) and \(z\). This model was originally conceived to study the fluorescence decay of the amino acid tryptophan [33].

The magnitude of the apparent dipole moment \(M\) is related to the factor \(\gamma\) by the following equation.

\[
y = \frac{64n^4}{3h} |M|^2 \tag{7}
\]

where \(h\) is Planck’s constant.

It is reasonable to assume \(M\) is parallel to one of the three ellipsoid axes \(x, y\) or \(z\) and so in making this assumption equation (6) can be simplified to:

\[
k_r \frac{v_{em}^2}{\nu} = \frac{\gamma n_0^5}{[n_0^2 + (n_1^2 - n_0^2)L_M]^2} \tag{8}
\]

where \(L_M\) is given by equation (9).

\[
L_M = \cos^2 \theta_x L_x + \cos^2 \theta_y L_y + \cos^2 \theta_z L_z \tag{9}
\]

The molecules in this study are assumed to be in an empty ellipsoidal cavity which takes the shape of the molecule.
and are surrounded by the solution of refractive index \( n_0 = n \). If this is the case, the electronic transition dipole moment, \( \mu = M, L_\mu = L_\mu \) and the refractive index of the molecule \( n_1 = 1 \) \[33\]. Equation (8) can hence be simplified further to equation (10).

\[
\sqrt{\frac{n^2}{k_r} n_{em}^3} = n^2 + P2
\]

\[ (10) \]

Such that

\[
P1 = \frac{8\pi^3|\mu|}{(1 - L_\mu)\sqrt{3h}}
\]

\[ (11) \]

\[
P2 = \frac{L_\mu}{1 - L_\mu}
\]

\[ (12) \]

The value of \( L_\mu \) can vary between 0 and 1 and indicates the shape of the molecule. A value of 1/3 denotes a spherical molecule while a value greater than 1/3 indicates that the molecule is an ellipsoid with its transition dipole moment along the shortest dimension and a value less than 1/3 denotes an ellipsoidal molecule with its transition dipole moment along the longest dimension.

Here, we extend the work by Toptygin et al. and focus on the fluorescence decays of two organic fluorescent dyes, PM546 and rhodamine 123, the structure of which are shown in figure 1, in solvents of different refractive indices. From these measurements, we calculate their transition dipole moment and ellipticity \( L_\mu \). The fluorescence lifetime of a fluorophore can be determined directly with high accuracy and precision using appropriate measurement techniques, e.g. time-correlated single photon counting [5].

PM546 and rhodamine 123 are fluorophores of particular interest because they are very photostable dyes with high quantum yields making them a popular choice for fluorescence applications. Their fluorescence decays are monoeXponential, which make them suitable for the fluorescence lifetime work performed here. PM546 is uncharged, and fluorescent dyes based on boron dipyrromethene (BODIPY), first synthesized in 1968, are small, bright, have high extinction coefficients and fluorescence quantum yields, low triplet yields, narrow absorption and emission spectra, have high two-photon excitation cross sections [34], are relatively photostable, can be easily synthesized and are available with different spectral characteristics and other chemical modifications [35–40]. They are used as laser dyes [41], viscosity sensors [42–46], hypochlorous acid sensors [47] or as fluorescent labels in fluorescence microscopy [48].

Rhodamine 123 is a positively charged (cationic) dye, stains mitochondria, which have a large membrane potential, and is frequently used in fluorescence microscopy [49] and single molecule spectroscopy [50]. It has been reported to have a high quantum yield of 90% and its emission is at longer wavelengths compared to BODIPY, as shown in figure 2.

2. Experimental section

Pyromethene 546 (1,3,5,7,8-pentamethylpyromethene-difluoroborate complex, BODIPY, PM546) was obtained from Exciton (USA), and rhodamine 123 from Sigma-Aldrich (UK), and stock solutions were prepared in methanol at a concentration of 0.6 mM and 1.21 mM, respectively. To obtain solutions of varying refractive index, methanol was mixed with different ratios of glycerol and mixtures made from 0% up to 95% glycerol while maintaining the same concentration of the dye. The final dye concentration for the spectroscopic measurement was 2.44 \( \mu \)M for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Hitachi U-400 UV/Vis absorption spectrometer (Japan) with 2 nm slit width. A scan speed of 120 nm min\(^{-1}\) was used for PM546 and 14.5 \( \mu \)M for rhodamine 123. The absorption spectra were measured using a Perkin-Elmer LS-50B luminescence spectrometer (UK). The parameters for measuring PM546 were as follows: excitation wavelength of 465 nm, scan speed of 120 nm min\(^{-1}\) and a slit width of 3 nm. For rhodamine 123 an excitation wavelength of 475 nm was used with a scan speed of 150 nm min\(^{-1}\) and a slit width of 7 nm. The refractive indices of the solutions were measured using
Fluorescence lifetime measurements were carried out using a Hamamatsu PLP-10 pulsed diode laser (Japan) at 470 nm, 10 MHz excitation rate and a 100 ns time window (for PM546) or 20 MHz and 50 ns time window (for rhodamine 123). The laser was coupled into a Leica, TCS SP2 inverted confocal scanning microscope (Germany), and time-correlated single photon counting (TSCPC) was carried out with a Becker and Hickl SPC-830 TCSPC module (Germany). The filter used for rhodamine 123 is a Semrock FF01-550/88-25 550 nm bandpass (USA), and for PM546 a Semrock FF01-514/30-25 514 nm filter (USA). Individual wells in a 96-well-glass-bottom multiwell plate were filled with 200 µl of the dye solutions and measured. The beam was focused in the middle of the well containing the sample solution with a Leica HC PL Fluotar 2× NA0.5 air objective.

3. Results and discussion

3.1. Fluorescence spectra

The normalised absorption and emission spectra of PM546 and rhodamine 123 in solutions of methanol and glycerol (with glycerol at 10% and 90%) are shown in figure 2. The PM546 absorption peak is at 493.5 nm in methanol and moves up to 496 nm in glycerol, and its emission peak is at 505 nm in methanol and 508.5 nm in glycerol. The rhodamine 123 absorption maximum is at 507 nm in methanol and 512 nm in glycerol, and its emission peak at 532 nm in methanol and 538 nm in glycerol.

3.2. Fluorescence decays

The normalized fluorescence decays of PM546 and rhodamine 123 in the varying refractive index media are shown in figure 3. A monoexponential decay function was used to fit the fluorescence decay curves, and the fits were good as judged by the flat residuals and goodness-of-fit χ²-values (not shown). The lifetime of PM546 in methanol is 5.64 ns, in good agreement with [41] where 5.58 ns is quoted. Rhodamine 123 has a lifetime of 3.98 ns in methanol, and the lifetimes of both dyes decrease when the refractive index increases by increasing the glycerol concentration, as expected.

3.3. Data analysis according to Hirayama’s empirical n² model

A plot of the inverse fluorescence lifetimes of PM546 and rhodamine 123 versus the square of the refractive index—according to the refractive index dependence of the Strickler—Berg formula equation (2)—is shown in figures 4(a) and (c). It can be seen that the data points follow a linear trend as expected. The rate constants can therefore be calculated through a plot based of the rearrangement of equation (1), making explicit use of the empirical n² dependence of the radiative rate constant k_r as suggested by Hirayama [29, 30]

\[
\frac{1}{\tau_f} = k_r + k_{nr} = k_{r0}n^2 + k_{nr}
\]  

where \( k_{r0} \) represents the value of \( k_r \) in vacuum.

For a straight line fit of the PM546 lifetime data in figure 4(a), assuming that \( k_{nr} \) is constant, \( k_{nr} \) was found to be \((6.36 \pm 0.35) \times 10^7\text{ s}^{-1}\), and the value of \( k_{r0} \) found to be \((6.37 \pm 0.18) \times 10^7\text{ s}^{-1}\). These values are in agreement with independent lifetime and quantum yield measurements of the same dye by López Arbeloa et al. [41] in many different solvents over a slightly larger refractive index range, plotted in [45].
Continuing the interpretation according to the $n^2$ dependence, for a refractive index of $n = 1.33$, the quantum yield, $\Phi$, is hence calculated to be $0.64 \pm 0.04$ from the equation:

$$\Phi = \frac{k_r}{k_r + k_{nr}}$$  \hspace{1cm} (14)

The thus calculated quantum yield of 0.64 however is below the value of 0.95 at a refractive index of 1.33 found in the literature for this dye in methanol [41]. Calculating the quantum yield from

$$\Phi = \frac{1}{\tau} k_r$$  \hspace{1cm} (15)

also yields a value of 0.64 which is, again, well below the literature value.

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**Figure 3.** The normalised fluorescence decays of (a) PM546 and (b) rhodamine 123 in mixtures of methanol and glycerol. The percentage values indicate the volume percentage of glycerol in solution. A mono-exponential decay function was fitted to the fluorescence decay curves, and the fits were good as judged by the flat residuals and goodness-of-fit $\chi^2$-values.

**Figure 4.** The inverse fluorescence lifetime $\tau^{-1}$ of (a) PM546 and (b) the radiative rate constant $k_r$ of PM546 plotted versus the square of the refractive index, $n^2$, of the methanol/glycerol mixtures. (c) The inverse fluorescence lifetime $\tau^{-1}$ of rhodamine 123 and (d) the radiative rate constant $k_r$ of rhodamine 123 plotted versus the square of the refractive index, $n^2$, of the methanol/glycerol mixtures.
The radiative rate constant of PM546 calculated from the spectra based on the Strickler–Berg equation (2) yields a higher value for $k_0$ of $(1.47 \pm 0.06) \times 10^8$ s$^{-1}$, i.e. significantly higher than the gradient of the $k_i$ versus $n^2$ plot based on Hirayama’s model.

One of the reasons for the underestimation could be due to the non-radiative rate constant $k_{nr}$ not being constant across the methanol/glycerol mixtures. We have therefore plotted $k_i$ versus $n^2$ which should yield a straight line through zero, without an intercept; according to equation (2). This is shown in figure 4(b). Although the data obey a linear relationship, a straight line fit through the origin is a poor fit. The gradient of $(9.47 \pm 0.06) \times 10^7$ s$^{-1}$ is higher than that obtained by the fit in figure 4(a), but still below the $k_0$ value given by the spectra. The best straight line fit of the data yields a non-zero intercept of $(6.29 \pm 0.35) \times 10^7$ s$^{-1}$ with a gradient of $(6.31 \pm 0.18) \times 10^5$ s$^{-1}$. However, a non-zero intercept is not envisaged in the Hirayama model. Furthermore, using equation (4), the dipole moment $M = \mu$ is calculated to be $(3.4 \pm 0.1) \text{ D}$. This is also well below the literature value of $6.93$ [41, 52] or $6.8$ [35]. Thus, while the conceptual qualitative straight-line interpretation works, the detailed quantitative analysis according to this model does not.

The data analysis for rhodamine 123 yields similar discrepancies. The fluorescence lifetime obtained from the experiments plotted versus the squared refractive index is shown in figure 4(c). The experimental results follow a straight line according to equation (2), except for a little bend occurring at high containing glycerol solutions. These data points are neglected for the fit. A straight line to fit the plot yields a gradient of $(1.13 \pm 0.02) \times 10^8$ s$^{-1}$ with an intercept of $(5.05 \pm 0.38) \times 10^7$ s$^{-1}$. The intercept value means the non-radiative rate constant is almost the same among the solutions, and the gradient value is $k_0$ according to equation (13). The radiative rate constant plotted versus the squared refractive index is shown in figure 4(d). The dashed line indicates a straight-line fit of the data points through the origin. The gradient is $(1.19 \pm 0.01) \times 10^8$ s$^{-1}$. The grey line is a straight-line fit with a non-zero intercept. The gradient is $(8.62 \pm 0.26) \times 10^7$ s$^{-1}$ and the intercept is $(6.55 \pm 0.51) \times 10^7$ s$^{-1}$. Again, an intercept has no meaning in the context of equation (2). A comparison of the gradient with the relevant term in the Strickler–Berg formula, $(1.510 \pm 0.002) \times 10^8$ s$^{-1}$, shows that it is too small.

Therefore, instead of using the empirical $n^2$ dependence suggested by Hirayama, we use Toptygin’s model given by equation (10) [33] to quantitatively analyze the data.

3.4. Data analysis according the Toptygin model
To calculate the transition dipole moment, $\mu$, from the model by Toptygin et al, equation (10), a plot of $(n^2 \chi_{em^2}/k_i)^{1/2}$ versus $n^2$ is shown in figure 5. From equations (10)–(12), $P_1$ and $P_2$ for PM546 are found to be $(4.92 \pm 0.06) \times 10^{-3}$ cm$^{3/2}$ and $(0.24 \pm 0.02)$ respectively. Re-arranging equation (11), this yields a transition dipole moment, $\mu$, of $7.1 \pm 1.1$ Debye (D) for PM546, more than twice the value obtained from the previous analysis with equation (4). The value of $(7.1 \pm 1.1)$ D is in excellent agreement with the literature value which has been found to vary from 6.8–6.93 D [35, 41, 52].

In addition, the depolarisation factor $L_\mu$, calculated from equation (12), is $0.19 \pm 0.03$. A value of $L_\mu < 1/3$ indicates that PM546 is an ellipsoidal molecule and its dipole moment along its longest axis. These findings are consistent with what is found in the literature for pyrromethene dyes. The direction of the $S_0 \rightarrow S_1$ transition dipole moment is perpendicular to that of the changes in the permanent electric dipole moments [51, 53, 54].

Furthermore the oscillator strength ($f$) of the transition $1 \leftarrow 0$ can be calculated from the transition dipole moment $\mu$ using equation (16) [55].

$$|\mu|^2 = \frac{3\hbar c^2}{8\pi^3 m_e c^2 f}$$  (16)
where $\nu$ is the wavenumber of the transition between the ground and excited state, $m_e$ is the electron mass, $h$ is Planck's constant, $c$ is the speed of light in a vacuum and $\epsilon$ the electron charge. Using the experimental value $\mu = 7.1$, $f$ is found to be 0.453. In previous studies the value of $f$ has been found to be $-0.42$ for PM546 depending on the details of the density functional theory (DFT) model used [35, 52].

For rhodamine 123, the values of $P_1$ and $P_2$ calculated from the gradient and the intercept of the plot are $(6.43 \pm 0.10) \times 10^{-3} \text{ cm}^{3/2} \text{s}^{-1/2}$ and $0.42 \pm 0.03$, respectively. From the $P_1$ and $P_2$ values obtained from the intercept and gradient, the value of $\mu$ is found to be $(8.1 \pm 0.1) \text{ D}$, and $L_0$ is found to be $0.29 \pm 0.07$. Thus, the intrinsic electronic transition dipole moment is $8.1 \text{ D}$ along the longest axis of the rhodamine 123 fluorophore, and its shape is ellipsoid. The data analysis based on Toptygin's model is summarised in Table 1.

We also tried to apply the formalism of plotting $(n^2 \nu^3 \epsilon_0^2 \mu_0^{-1})^{1/2}$ versus $n^2$ to BODIPY-C12, a fluorescent molecular rotor [44, 45]. However, the radiative rate constant is much smaller in comparison to the non-radiative decay rate in BODIPY-C12 and hence extracting the radiative decay constant from the data with sufficient accuracy proved difficult. The data points show too much scatter, and the standard deviations of the gradient and intercept are too large to be meaningful [56].

Investigating Nile red, a dye with more complicated photophysics than the single state systems investigated here [57] also did not work: The average lifetime gets longer as glycerol is added, and the plots of $(n^2 \nu^3 \epsilon_0^2 \mu_0^{-1})^{1/2}$ versus $n^2$ are not linear [56]. Analysing the average lifetime of GFP fluorescence decays as a function of the refractive index (from [10]) in this manner yielded reasonable values, but their interpretation is difficult as the GFP fluorescence decays multi-exponentially.

### 4. Conclusion

From fluorescence lifetime measurements of the fluorescent dyes PM546 and rhodamine 123 in solvents of different refractive index, we have been able to calculate their transition dipole moments, using a model proposed by Toptygin et al [33]. The results are in excellent agreement with those from the literature. The transition dipole moment of PM546 is found to be $(7.1 \pm 1.1) \text{ D}$ when using the Toptygin model. Conversely the result of $3.4 \text{ D}$ calculated from Hirayama's empirical $n^2$ dependence is in poor agreement with that of the literature, hence showing the limitations of this empirical relationship. The Strickler–Berg equation, equation (2), is appropriate when it is used for the purpose for which it was intended: to calculate the radiative decay rate $k_r$ of a dye in a medium of a given refractive index $n$ from the fluorescence emission spectrum $F(\nu)$ and the extinction coefficient spectrum $\varepsilon(\nu)$ measured in the same medium. However, if the refractive index $n$ is changing, then not only $k_r$, but also $\varepsilon(\nu)$ varies with the refractive index, and therefore the Strickler–Berg equation cannot be used to predict the variation of $k_r$ with $n$ by simply plotting $k_r$ versus $n^2$. However, the empirical relationship between the inverse lifetime and the radiative rate constant and the square of the refractive index is still a linear one, and as such still suitable as an empirical calibration to convert lifetime into a refractive index or vice versa. In combination with FLIM, it is a viable contrast mechanism [13, 14, 25, 26].

The approach presented here is important for quantitative understanding further which molecular probes are suited to which applications and being able to predict the behaviour of new or important dyes like PM546 in different environments, for example fluorogen-based reporters [58], quantum dots [17], novel genetically encoded fluorescent proteins [59] or dyes, particularly for fluorescence imaging. TCSPC is a very accurate way of directly measuring fluorescence lifetimes, without the use of a standard or reference. It allows for a higher precision than fluorescence quantum yield determinations, and so this novel method by Toptygin et al provides a potentially highly accurate method for measuring transition dipole moments based on fluorescent lifetimes and emission wavelength. No knowledge of the cavity radius is required, as, for example, in the Lippert equation. Furthermore, the transition dipole moment of rhodamine 123 is found to be $(8.1 \pm 0.1) \text{ D}$. We have also shown one of the limitations of this model by investigating a molecule that is sensitive to solvent viscosity, such as BODIPY-C12 [44, 45] or polarity, such as Nile red [57]. Here we see that this method is unable to predict the electronic transition moment accurately due to the large impact of viscosity on the non-radiative rate constant, or multi-exponential fluorescence decays and the non-linearity of average lifetime plots according to equation (10) [56]. It is of value to understand these limitations of both methods so that we can better characterise fluorescent probes in the future.

**Table 1.** The transition dipole moment of PM546 and rhodamine 123 as obtained by fluorescence lifetime measurements in solvents of varying refractive index. The figures in bold denote the results from Toptygin’s model.

| Gradient/ cm$^{-3/2}$ s$^{-1/2}$ | Intercept | $P_1 \times 10^{-3}$ cm$^{3/2}$ s$^{-1/2}$ | $P_2$ | $[\varepsilon](\text{D})$ | $L_0$ |
|-----------------------------|------------|---------------------------------|--------|-----------------|-------|
| PM546                      | 203.2 ± 2.4 | 48.1 ± 4.8                      | 4.92 ± 0.06 | 0.24 ± 0.02 | 7.1 ± 1.1 |
| Rhodamine 123              | 155.6 ± 2.5 | 65.1 ± 4.9                      | 6.4 ± 0.1 | 0.42 ± 0.03 | 8.1 ± 0.1 |

The results are in excellent agreement with those from the literature. The transition dipole moment of PM546 is found to be $(7.1 \pm 1.1) \text{ D}$ when using the Toptygin model. Conversely the result of $3.4 \text{ D}$ calculated from Hirayama’s empirical $n^2$ dependence is in poor agreement with that of the literature, hence showing the limitations of this empirical relationship. The Strickler–Berg equation, equation (2), is appropriate when it is used for the purpose for which it was intended: to calculate the radiative decay rate $k_r$ of a dye in a medium of a given refractive index $n$ from the fluorescence emission spectrum $F(\nu)$ and the extinction coefficient spectrum $\varepsilon(\nu)$ measured in the same medium. However, if the refractive index $n$ is changing, then not only $k_r$, but also $\varepsilon(\nu)$ varies with the refractive index, and therefore the Strickler–Berg equation cannot be used to predict the variation of $k_r$ with $n$ by simply plotting $k_r$ versus $n^2$. However, the empirical relationship between the inverse lifetime and the radiative rate constant and the square of the refractive index is still a linear one, and as such still suitable as an empirical calibration to convert lifetime into a refractive index or vice versa. In combination with FLIM, it is a viable contrast mechanism [13, 14, 25, 26].

The approach presented here is important for quantitative understanding further which molecular probes are suited to which applications and being able to predict the behaviour of new or important dyes like PM546 in different environments, for example fluorogen-based reporters [58], quantum dots [17], novel genetically encoded fluorescent proteins [59] or dyes, particularly for fluorescence imaging. TCSPC is a very accurate way of directly measuring fluorescence lifetimes, without the use of a standard or reference. It allows for a higher precision than fluorescence quantum yield determinations, and so this novel method by Toptygin et al provides a potentially highly accurate method for measuring transition dipole moments based on fluorescent lifetimes and emission wavelength. No knowledge of the cavity radius is required, as, for example, in the Lippert equation. Furthermore, the transition dipole moment of rhodamine 123 is found to be $(8.1 \pm 0.1) \text{ D}$. We have also shown one of the limitations of this model by investigating a molecule that is sensitive to solvent viscosity, such as BODIPY-C12 [44, 45] or polarity, such as Nile red [57]. Here we see that this method is unable to predict the electronic transition moment accurately due to the large impact of viscosity on the non-radiative rate constant, or multi-exponential fluorescence decays and the non-linearity of average lifetime plots according to equation (10) [56]. It is of value to understand these limitations of both methods so that we can better characterise fluorescent probes in the future.
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References

[1] Birch D J S 2011 Fluorescence detections and directions Meas. Sci. Technol. 22 052002
[2] Shi W, Li X and Ma H 2014 Fluorescent probes and nanoparticles for intracellular sensing of pH values Methods Appl. Fluoresc. 2 042001
[3] Sun S, Ungerböck B and Mayr T 2015 Imaging of oxygen in microreactors and microfluidic systems Methods Appl. Fluoresc. 3 034002
[4] Kawanabe S, Araki Y, Uchimura T and Imasaka T 2015 Methods Appl. Fluoresc. 3 034002
[5] Becker W 2012 Fluorescence lifetime imaging—techniques and applications J. Microsc. 247 119 – 36
[6] Borst J W and Visser A J W G 2010 Fluorescence lifetime imaging microscopy in life sciences Meas. Sci. Technol. 21 102002
[7] Berezin M Y and Acheile S 2010 Fluorescence lifetime measurements and biological imaging Chem. Rev. 110 2641 – 84
[8] Suhling K et al 2015 Fluorescence lifetime imaging (FLIM): basic concepts and some recent developments Med. Photonics 27 3 – 40
[9] Toptygin D 2003 Effects of the solvent refractive index and extinction coefficient of a fluorescent solute J. Fluoresc. 13 201 – 19
[10] Suhling K, Siegl J, Phillips D, French P M W, Lévêque-Fort S, Webb S E D and Davis D M 2002 Imaging the environment of green fluorescent protein Biophys. J. 83 3589 – 95
[11] Borst J W, Hink M A, Hoek A and Visser A J W G 2005 Effects of refractive index and viscosity on fluorescence and anisotropy decays of enhanced cyan and yellow fluorescent proteins J. Fluoresc. 15 153 – 60
[12] Söhnle A C, Kohl W, Gregor I, Enderlein I, Rieger B and Busch K B 2016 Probing of protein localization and shuttling in mitochondrial microcompartments by FLIM with sub-diffraction resolution Biochim. Biophys. Acta 1857 1290 – 9
[13] Treanor B, lanigan P M, Suhling K, Schreiber T, Munro I, Neil M A, Phillips D, Davis D M and French P M W 2005 Imaging fluorescence lifetime heterogeneity applied to GFP-tagged MHC protein at an immunological synapse J. Microsc. 217 36 – 43
[14] Tregidgo C, Levitt J A and Suhling K 2008 Effect of refractive index on the fluorescence lifetime of green fluorescent protein J. Biomed. Opt. 13 031028
[15] Zhao M, Li Y and Peng L L 2014 FPGA-based multi-channel fluorescence lifetime analysis of fluorophores multiplexed frequency-sweeping lifetime imaging Opt. Express 22 23073 – 85
[16] Schnippe H and Sandoghdar V 2002 Spontaneous emission of europium ions embedded in dielectric nanospheres Phys. Rev. Lett. 89 257403
[17] Wüster S F, de Mello Donega C and Mejerink A 2004 Local-field effects on the spontaneous emission rate of CdTe and CdSe quantum dots in dielectric media J. Chem. Phys. 121 4310 – 5
[18] Khalid A, Chung K, Rajasekharan R, Lau D W M, Karle T J, Gibson B C and Tomljenovic-Hanic S 2015 Lifetime reduction and enhanced emission of single photon color centers in nanodiamond via surrounding refractive index modification Sci. Rep. 5 11179
[19] Ma Y J, Rajendran P, Blum C, Cesa Y, Gartner M N, Bruhwiler D, Subramaniam V 2011 Microspectroscopic analysis of green fluorescent proteins infiltrated into mesoporous silica nanochannels J. Colloid Interface Sci. 356 123 – 30
[20] Mohanty J and Nau W M 2005 Ultrafast rhodamine with cucurbituril Angew. Chem., Int. Ed. 44 5750 – 4
[21] Toptygin D and Brand L 1993 Fluorescence decay of DPH in lipid membranes: influence of the external refractive index Biophys. Chem. 48 203 – 20
[22] Krishna M M G and Periasamy N 1998 Fluorescence of organic dyes in lipid membranes: site of solubilization and effects of viscosity and refractive index on lifetimes J. Fluoresc. 8 81 – 91
[23] Joosen L, Hink M A, Gadella T W J and Goedhart J 2014 Effect of fixation procedures on the fluorescence lifetimes of Aequorea victoria derived fluorescent proteins J. Microsc. 256 166 – 76
[24] Ganguly S, Clayton A H A and Chattopadhyay A 2011 Fixation alters fluorescence lifetime and anisotropy of cells expressing EYFP-tagged serotinin (1A) receptor Biochem. Biophys. Res. Commun. 405 234 – 7
[25] van Manen J H, Verkuipen P, Wittenborg P, Subramaniam V, van den Berg T K, Roos D and Otto C 2008 Refractive index sensing of green fluorescent proteins in living cells using fluorescence lifetime imaging microscopy Biophys. J. 94 L67 – 9
[26] Pflüger A, Zhao L L, Ohulchansky T Y, Qu J L, Land Prasad P N 2012 Fluorescence lifetime of fluorescent proteins as an intracellular environment probe sensing the cell cycle progression ACS Chem. Biol. 7 1385 – 92
[27] Gohar A V, Cao R F, Jenkins P, Li W Y, Houston J P, Houston K D 2013 Subcellular localization–dependent changes in EGF/P fluorescence lifetime measured by time-resolved focus cytometry Biomed. Opt. Exp. 4 1390 – 400
[28] Stricker S J and Berg R A 1962 Relationship between absorption intensity and fluorescence lifetime of molecules J. Chem. Phys. 37 814 – 20
[29] Hirayama S and Phillips D 1980 Correction for refractive index in the comparison of radiative lifetimes in vapour and solution phases J. Photochem. 12 139 – 45
[30] Hirayama S, Yasuda H, Okamoto M and Tanaka F 1991 Effect of pressure on the natural radiative lifetimes of anthracene derivatives in solution J. Phys. Chem. 95 2971 – 5
[31] Lewis J E and Maroncelli M 1998 On the (un)interesting dependence of the absorption and emission transition moments of coumarin 153 on solvent Chem. Phys. Lett. 282 197 – 203
[32] Breffke J, Williams B W and Maroncelli M 2015 The photophysics of three naphthylmethylene malononitriles J. Phys. Chem. B 119 9254 – 67
[33] Toptygin D, Savtchenko R S, Meadow N D, Roseman S and Brand L 2002 Effect of the solvent refractive index on the excited-state lifetime of a single tryptophan residue in a protein J. Phys. Chem. B 106 3724 – 34
[34] Xu C and Webb W B 1996 Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm J. Opt. Soc. Am. B 13 481 – 91
[35] Schmitt A, Hinkeldey B, Wild M and Jung G 2009 Synthesis of the core compound of the BODIPY dye class: 4,4′-Difluoro-4-bora-(3a,4a)-diazas-s-indacene J. Fluoresc. 19 755 – 8
[36] Hinkeldey B, Schmitt A and Jung G 2008 Comparative photostability studies of BODIPY and fluorescein dyes by using fluorescence correlation spectroscopy Chemphyschem 9 2019 – 27
[37] Kee H L et al 2005 Structural control of the photodynamics of boron-dipyrrin complexes J. Phys. Chem. B 109 20453 – 43
[38] Ulrich G, Ziesel R and Harriman A 2008 The chemistry of fluorescent bodipy dyes: versatility unsurpassed Angew. Chem., Int. Ed. 47 1184 – 201
[39] Boens N, Leen V and Dehaen W 2012 Fluorescent indicators based on BODIPY Chem. Soc. Rev. 41 1130 – 72
[40] Benniston A C and Copley G 2009 Lighting the way ahead with boron dipyrromethene (Bodipy) dyes Phys. Chem. Chem. Phys. 11 4124–31
[41] López Arbeloa F, López Arbeloa T and López Arbeloa I 1999 Electronic spectroscopy of pyrromethene 546 J. Photochem. Photobiol. A 121 177–82
[42] Benniston A C, Harriman A, Whittle V L and Zelzer M 2010 Molecular rotors based on the boron dipyrromethene fluorophore Eur. J. Org. Chem. 20 4024–30
[43] López Arbeloa F, López Arbeloa T and López Arbeloa I 1999 Electronic spectroscopy of pyrromethene 546 J. Photochem. Photobiol. A 121 177–82
[44] Benniston A C, Harriman A, Whittle V L and Zelzer M 2010 Molecular rotors based on the boron dipyrromethene fluorophore Eur. J. Org. Chem. 20 4024–30
[45] Kuimova M K, Yahioglu G, Levitt J A and Suhling K 2008 Molecular rotor measures viscosity of live cells via fluorescence lifetime imaging J. Am. Chem. Soc. 130 6672–3
[46] Levitt J A, Kuimova M K, Yahioglu G, Chung P H, Suhling K and Phillips D 2009 Membrane-bound molecular rotors measure viscosity in live cells via fluorescence lifetime imaging J. Phys. Chem. C 113 11634–42
[47] Yu S-Y, Blaya S and Carretero L 2003 Ab initio study of absorption and emission spectra of PM567 Chem. Phys. Lett. 374 206–14
[48] Calzaferri G and Rytz R 1995 Electronic-transition oscillator strength by the extended Hückel molecular-orbital method J. Phys. Chem. 99 12141–50
[49] Chung P-H 2012 Advanced fluorescence lifetime imaging and spectroscopy techniques for biological samples PhD Thesis, Department of Physics, King’s College London, London
[50] Levitt J A, Chung P H and Suhling K 2015 Spectrally resolved fluorescence lifetime imaging of Nile red for measurements of intracellular polarity J. Biomed. Opt. 20 096002
[51] Jullien L and Gautier A 2015 Fluorogen-based reporters for fluorescence imaging: a review Methods Appl. Fluoresc. 3 042007
[52] Hoepker A C, Wang A, Le Marois A, Suhling K, Yan Y and Marriott G 2015 Genetically encoded sensors of protein hydrodynamics and molecular proximity Proc. Natl Acad. Sci. USA 112 E2569–74