Two- and multiphoton excitation of conjugate-dyes using a continuous wave laser

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Abstract

We discuss the possibility of obtaining a fluorescence signal depending nonlinearly on the excitation intensity by inhibiting the fluorescence energy transfer in a conjugate-pair dye molecule and its application in scanning microscopy. The dynamical behaviour of the conjugate dye molecule and the nonlinear behaviour of the fluorescence intensity is analysed. The benefits for scanning fluorescence microscopy are outlined.

1. Introduction

The use of two-photon excited fluorescence is one of the most important developments in scanning light microscopy during the last few years [1–3]. One of the benefits of two-photon excitation is the possibility of generating three-dimensional images without a spatial filter, i.e. confocal detection pinhole, in front of the detector. Standard two-photon excitation is performed by the simultaneous absorption of two photons of half the energy required for excitation of the fluorophore. Unfortunately, as a result of the low cross-section, two-photon absorption via a virtual state is less probable as its single photon counterpart, so that powerful lasers are required for fast image acquisition. It is well known that the two-photon excitation process can be enhanced by involving an intermediate state of the dye molecule in which case the resolution could also be improved [4]. However, it might prove that the number of dyes featuring suitable intermediate steps with subsequent two-photon induced fluorescence is limited. It is therefore interesting to investigate on alternative approaches that use low-power light sources for generating a two-photon imaging mode with the fluorescence signal in the visible range. Such an approach is described in this paper. It is based on fluorescence resonant energy transfer in a conjugate dye consisting of two molecules. Energy transfer has been first described by Förster [5] and is a well investigated phenomenon. It is widely applied both in fluorescence spectroscopy and microscopy. Fluorescence energy transfer can be used as a "molecular ruler" to determine the distance between two molecules. Excellent accounts of the mechanisms and benefits of fluorescence energy transfer in microscopy, spectroscopy and molecular biology can be found, for instance, in the review paper by Clegg [6], and the...
volume by Lakowicz [7]. A recent application is given in Ref. [8]. Here, we give rather a brief summary of fluorescence energy transfer in order to outline the basic ideas of this paper.

2. Theory

Fluorescence energy transfer, as first described by Förster [5], is the transfer of the excited state energy from a donor molecule to an acceptor molecule. This transfer takes place without the appearance of a photon and is a result of a direct dipole–dipole interaction at a close spatial distance between the molecules. In principle, both donor and acceptor molecules can be fluorescent so that, both donor and acceptor can independently absorb and emit photons. The standard mode of using a donor-acceptor pair is as follows. The donor absorbs a photon, and then the donor can either relax internally, or transfer the energy to the acceptor dye, which is then excited. The energy transfer rate is significant when the dipole–dipole interaction is strong enough as to compete with the internal relaxation, e.g. fluorescence, thermal quenching of the donor. After receiving the energy from the donor, the acceptor can relax either through spontaneous emission or by thermal relaxation. As a result of the energy transfer, the donor life time is reduced. For a total energy transfer, i.e., for a transfer rate converging to unity, the life time of the excited state of the acceptor becomes infinitely short.

The diagram of fluorescence energy transfer is presented in Fig. 1. As the energy transfer probability never reaches unity, the donor molecules have a non-vanishing probability to fluoresce. A donor fluorescence rate marked as $k_{fd}$ (Fig. 1) has to be considered. The donor fluorescence increases when the acceptor molecule is in the excited state. When excited, the acceptor molecule cannot accept the energy from the donor, the energy transfer is inhibited, thus increasing the fluorescence rate $k_{fd}$. Therefore, the fluorescence signal due to the inhibition of the energy transfer depends on the consecutive absorption of two photons whose energies are needed for the excitation of the acceptor and the donor respectively. This photon-optics viewpoint suggests that this signal depends on the product of the power used for the excitation of the donor and acceptor molecules [9].

The efficiency of the energy transfer depends on the distance of the two dye molecules and their properties. The energy transfer rate to the acceptor molecule is given by

$$k_{et} = \frac{1}{\tau_d} \left( \frac{R_0}{R} \right)^6,$$

where $\tau_d$ is the life time of the donor in the absence of the acceptor. $R$ is the distance between the molecules and $R_0$ the distance at which the probability for energy transfer is equal to spontaneous emission of the donor dye, i.e. the Förster distance. $R_0$ in Ångströms is given by

$$R_0 = 9.7 \times 10^3 \left( \frac{K^2 Q}{n^4} \right) \text{Å},$$

$K^2$ being the dipole–dipole orientation factor, $Q$ the donor quantum yield without the acceptor, $n$ the refractive index of media and $J$ the overlapping integral of the donor emission and acceptor extinction spectra defined by

$$J = \frac{\int F(\lambda) \varepsilon(\lambda) \lambda^4 \, d\lambda}{\int F(\lambda) \, d\lambda},$$
where $\lambda$ is the wavelength, $F(\lambda)$ the normalised emission spectrum of the donor and $\varepsilon(\lambda)$ the extinction of the acceptor. $R_0$ is typically around 50 Å in water.

3. Nonlinear dependence of fluorescence from the donor dye in a donor-acceptor system with inhibited energy transfer

Observing the fluorescence from the donor $k_{fd}$ (Fig. 1), it becomes clear that the donor fluorescence can be increased by blocking the energy transfer channel ($k_e$). This blocking occurs if the acceptor is in the higher state which takes place either by saturating the acceptor via energy transfer, or by introducing a second laser beam with the purpose of directly exciting the acceptor. This additional fluorescence of the donor is a result of two independent photons absorbed by the conjugate dye. To model this behaviour we have made a state-diagram of the conjugate dye (Fig. 2). The capital letters denote the excited states of the donor (D) and the acceptor (A) molecules, whereas the small letters (a, d) denote the respectively relaxed states of the dye molecules. This diagram enables the formulation of the differential equations governing the system:

$$\frac{dN(da)}{dt} = -k_{exc}(1+p)N(da) + k_{fd}N(Da) + k_{fa}N(dA),$$

$$\frac{dN(Da)}{dt} = -k_{et}N(Da) + k_{et}N(da) + k_{fa}N(dA),$$

$$\frac{dN(DA)}{dt} = -k_{fd}N(DA) + k_{et}N(da) + k_{fa}N(dA),$$

$$\frac{dN(dA)}{dt} = -k_{et}N(dA) + k_{fd}N(DA) + k_{et}N(Da),$$

Fig. 2. Energy state diagram for defining the differential equations for the donor–acceptor system. 'da' means donor and acceptor not excited, 'Da' denotes donor in excited state and acceptor not excited, 'dA' is donor not excited and acceptor excited, and 'DA' is both donor and acceptor in excited state. The rates of excitation ($k_{exc}$) and others as in Fig. 1.

Fig. 3. Solutions for Eq. (4). The dotted curves present the donor fluorescence probability as a function of excitation rate ($k_{exc}$) with $p_{exc} = 5k_{exc}$. The energy transfer efficiencies were 0.9, 0.99, 0.999 and 0.9999 for the respective dotted curves. The solid curves present the fluorescence observed at the acceptor emission wavelength. Each of the donor fluorescence curves exhibits an area of nonlinear dependence on the excitation intensity. For the 0.9999 case the area spans over more than three orders of magnitude.
$k_{fd}$ and $k_{fa}$ the fluorescence rates of the donor and acceptor respectively and $k_e$ the rate of energy transfer. The solution of the differential equations (4) describes the behaviour of a conjugate dye for our purposes. In Fig. 3, the probability is plotted of the donor to be in the excited state as a function of the excitation rate $k_{exc}$ for different energy transfer efficiencies, $k_{exc}/(k_{exc} + k_{fd})$ of 0.9, 0.99, 0.999, and 0.9999. As the fluorescence of the donor is given by $k_{fd} N(Da) + k_{fa} N(DA)$ this expression describes the behaviour of the donor fluorescence as a function of excitation power. The population of the excited state of the acceptor molecule is also shown for comparison (solid line). The acceptor molecule was supposed to be excited at a rate of $5k_{exc}$. Apart from the region where the dye is saturated and the region where the excitation rate of the acceptor is low, the donor fluorescence generated by exciting the acceptor molecule has a pronounced nonlinear response. It is a quadratic dependence, in good approximation, and for the limiting case of equal excitation wavelengths of both molecules, an exact square-power law. For 99.99% energy transfer efficiency this square-power dependence spans over three orders of magnitude of excitation intensity. One can also notice that a linear component of the fluorescence signal is also present for low energy transfer rates. In this case, the nonlinear component can be extracted by special signal recording techniques such as second harmonic detection [11]. Fig. 3 suggests that a high energy transfer rate is needed for direct observation of the second order effect. In a conjugate dye with several acceptor dyes the order will be respectively the number of dyes (and photons) associated in the process [10].

In a preliminary experiment, we tested the possibility of increasing donor fluorescence signals by inhibiting the energy transfer through acceptor excitation. We chose a commercially available conjugate dye, Quantum Red by Sigma Chemicals, which has a large fluorescent protein (Phycoerythrin) as the donor and is covalently coupled, according to the manufacturer, to five to seven red acceptor molecules (CY5). The excitation of the donor and the acceptor was performed at 488 nm and 633 nm, respectively. Phycoerythrin features its peak fluorescence at around 580 nm. The 633 nm HeNe laser (Oriel Inc.) excited in turn the CY5 that emits primarily at around 670 nm. In normal use, Quantum Red is excited at 488 or 514 nm and the emission of CY5 is observed at 670 nm. As we were interested in the fluorescence of the donor, we observed the fluorescence at 580 nm of Phycoerythrin. In Fig. 4 the effect of the acceptor excitation is displayed. In this experiment, the 633 nm light was switched on and off. When switched on, the amount of observed fluorescence was instantaneously increased as can be seen from the first steep rise of the signal. In addition, a further long term increase in donor fluorescence was observed which we assume to be due to bleaching of the acceptor molecule. This conclusion is supported by the noticeable reduction of the fluorescence signal at the acceptor emission wavelength. Although the effect was well pronounced it was not possible to observe the intensity dependence of the signal in these preliminary experiments. We think this is due to the intense bleaching of this particular acceptor molecule, and the high power of the 633 nm laser that most probably saturated the acceptor molecule. Our theoretical calculations also suggest that with an energy transfer efficiency of about 0.9, the nonlinear dependence on the illumination power is difficult to observe in a steady-state-experiment. A perfect quadratic dependence requires that the excitation of both the acceptor and the donor are excited with the
same wavelength. This is the case when the excitation of the blocking acceptor molecules is excited via energy transfer from the donor only. If the excitation wavelengths are different, as in the previous example, the nonlinear term will depend on the product of the intensities of the two beams.

4. Conclusion

We have presented a method of generating a donor fluorescence signal in a donor-acceptor pair of fluorescence molecules, the dependence of which is nonlinear. This nonlinear dependence is a consequence of the fact that the signal is generated by the absorption of two photons at the same point in the focus. This view is supported by the evaluation of the differential equations of the fluorophores. In microscopy, this excitation mechanism should allow for three-dimensional imaging without spatial filtering in front of the detector. We cannot expect this method to limit bleaching to the focal region, as it is the case with two-photon excitation via a virtual state.

However, as the used excitation light is in the visible range, two-photon excitation of coupled energy-transfer dyes increases the resolution which is a distinct advantage over the virtual state two-photon excitation. The generation of the nonlinear fluorescence signal of the donor cannot take place if the acceptor molecule is able to absorb the transferred energy by switching to a higher singlet state. Absorption to a higher singlet state of the acceptor should be avoided by choosing appropriate donor-acceptor pairs. A clear challenge to using conjugate dyes is to find a conjugate dye pair with low donor fluorescence. This problem can be alleviated by modulating the excitation beam and observing the second harmonic of the donor fluorescence signal [11]. Another intriguing approach in using conjugate dyes is multiphoton detection as we have pointed out in an earlier publication [9]. Use of coincidence detection of both the acceptor and donor fluorescence suppresses the linear part of the donor fluorescence and provides for true two- and multiphoton detection.

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References