Experimental investigation of the Photophysics of Oregon Green 488 and its suitability to biological research applications

Advanced ITPL Practical Report

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Abstract: This report is a summary of experimental work carried out in the Department of Physics and Astronomy, UCL as part of the AITPL experimental course at CoMPLEX, UCL. Experiments to determine a number of photophysical characteristics of Oregon Green 488 (Life Technologies, UK). The emission and absorption spectrum were determined. The fluorescence lifetime and anisotropy in water were measured. The fractional depletion, change in anisotropy and change in lifetime achieved by stimulated emission was determined for both single and two photon excitation. The quantum yield and a two photon cross section spectrum were determined by comparison to similar, well characterised fluorescent molecules, fluorescein and rhodamine B. The results of these experiments are discussed in the context of quantitative biological research applications which will be made possible by the understanding of these physical characteristics.

Contents

Introduction

Fluorescence microscopy is ubiquitous in cell and molecular biology (for a review see Lichtman & Conchello, 2005 [1]). It enables specific, targeted spatial visualisation of biological molecules at high resolution with superior contrast to absorption based microscopy via typically non-invasive labelling with fluorescent dyes or genetically encoded fluorescent proteins [2]. Furthermore, detailed understanding of the photophysics of fluorescent molecules has resulted in a suite of experimental applications for biological research. These methods, when suitably employed, can convey quantitative information on bio-molecular structure and dynamics, bio-molecular environment and improved resolutions far beyond conventional microscopy [3].

The power and versatility of fluorescence techniques in biological research has resulted in a vast number of commercially produced fluorescent probes designed for various applications. Over 3000 fluorescent, bio-molecular labelling reagents for studying cell structure and function are outlined in the Molecular Probes Handbook, 11th edition available online (http://probes.invitrogen.com-/handbook/). Two such fluorescent probes are fluorescein and Oregon Green 488 (OG488). Fluorescein is a well characterised, synthetic organic compound whereas OG488, a derivative of fluorescein, is much less well characterised. Fluorescein has been widely used to label proteins for microscopy and flow cytometry due to its high quantum yield, high absorptivity, its water solubility and the fact its absorbance peak, at a wavelength of 494nm, closely matches the spectral line of readily available argon-ion lasers. OG488 is claimed to have similar properties to fluorescein with a number of advantages including pH insensitivity in physiological regimes, greater photo stability and less quenching (Life Technologies Website).

The aim of this work was to experimentally determine photophysical characteristics of Oregon Green such that it can be reliably used as a fluorescent probe in a variety of quantitative biological imaging modalities. The concentration dependence of the absorption spectra was investigated. Time correlated single photon counting (TCSPC) experiments in conjunction with a confocal microscope system were used to determine the continuous wave stimulated emission depletion (CW-STED) characteristics, the fluorescence lifetime and the anisotropy of OG488 in water. TCSPC at various concentrations was also performed allowing determination of the previously unpublished quantum yield of OG488. Finally, a two photon cross section spectrum of OG488 from 730nm to 890nm was determined.

Fluorescence

The emission of light from a molecule occurs when an electronically excited state relaxes to the ground state by emission of a photon [4][5]. When pairs of electrons in the excited state and ground state have opposing quantum mechanical spin the excitation is said to be a singlet state and the relaxation occurs on a timescale of the order of 10^{-9}s. This process of relaxation from a singlet state to the ground state by the emission of a photon is termed fluorescence. The energy difference, E, between the excited and ground states is converted to a photon of wavelength λ determined by Equation 1,

$$E = \frac{hc}{\lambda} \qquad 1$$

where h is Planck's constant and c is the speed of light. Conversely, an incident photon can be absorbed resulting in an electron transition from the ground state to an excited state, this process occurs on a timescale of the order of 10^{-15}s. If the energy of the absorbed or emitted photon is not exactly equal to the energy difference between the electronic ground and excited state this energy difference contributes to the vibrational and rotational energy of the molecule. In the time between excitation and spontaneous emission, the fluorescence lifetime, the molecule will undergo a

vibrational relaxation leading to a lower average energy for the emitted photon. This results in the Stokes shift, a shift in the emission spectrum of a fluorophore towards longer wavelengths compared to the absorption spectrum. Figure 1 is a Jablonski diagram representing some of the electronic energy states in a typical fluorescent molecule and some of the possible transitions between these states.

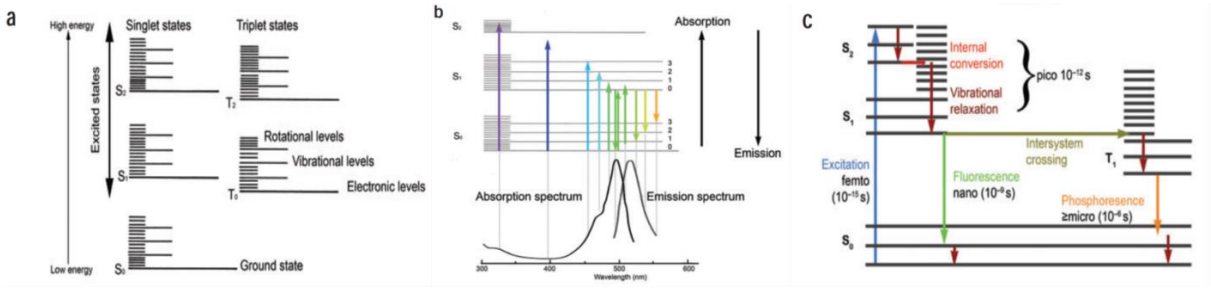

Figure 1: Jablonski diagrams A) Jabolnski diagram of the available energy states of a molecule including fluorescent Singlet states, non-fluorescent Triplet states and their associated rotational and vibrational energy states. B) How different electronic transitions relate to the wavelength of the absorbed or emitted photons, the peaks in the absorption and emission spectra indicate which transitions are most probable. C) Typical timescales for electronic transitions. Adapted from [1]

The specific configuration of the outer electronic orbitals define the typical fluorescence wavelengths and the molecule's efficiency as a fluorescent compound. For even the very simplest of molecules these orbitals and their behavior are almost impossible to accurately predict and as such empirical methods are the most reliable way to characterise the photophysics of these molecules.

Confocal Microscopy

An image of a fluorescently labeled sample can easily be reconstructed by scanning the focal spot of a laser across a sample and recording the fluorescence intensity at each point. The Stokes shift allows the shorter wavelength excitation to be almost completely filtered out by appropriate choice of dichroic mirrors and detector filters resulting in excellent contrast [1]. In 3D samples however an hourglass region of the sample centered on the focal plane is illuminated resulting in fluorescence originating from above and below the focal plane contributing to the image intensity. In confocal microscopy [6], a pinhole is used to eliminate out of focus light emitted from a sample and as such eliminates light from above and below the focal plane of the microscope objective. A 3D image can then be reconstructed by scanning across multiple planes sequentially.

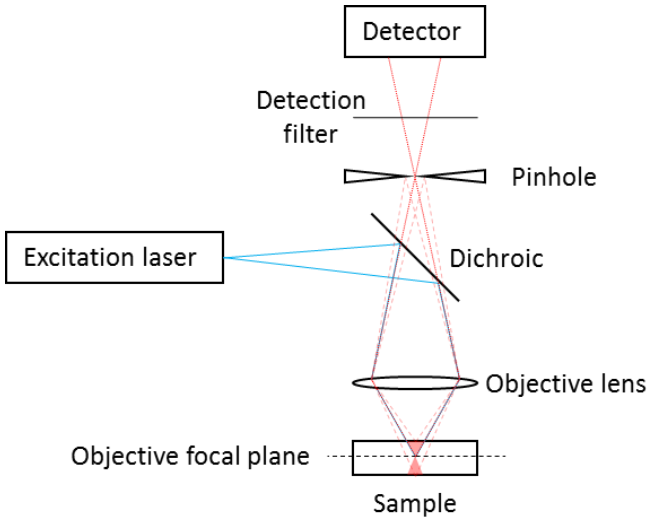

Figure 2: Diagram of a confocal microscope setup.

Two Photon Microscopy

Two photon microscopy is another laser scanning technique which is inherently confocal [7]. Two photon microscopy relies on the excitation of a fluorophore by the simultaneous (within approximately 0.5fs) absorption of two lower energy photons. This less probable transition is no longer proportional to the excitation intensity but to the excitation intensity squared. This means the excitation intensity can be tuned such that fluorescence is only possible in a small region close to the focal plane where the laser intensity is at its greatest. This achieves the same effect as using a pinhole with the added benefit that no unnecessary fluorescence occurs out of the focal plane which could otherwise result in bleaching. Furthermore, less scattering of longer wavelengths in biological samples means a deeper penetration can be achieved. Pulsed lasers which deliver their power in very short, intense, femto second pulses with MHz repetition rates are often used to instigate these low probability transitions. A Titanium Sapphire laser is commonly used as it is simpler to operate than mode-locked, cavity dumped lasers and can self mode-lock via the Kerr lens effect [4]. An active mode-locker can be included in the cavity to stabilise the free running mode-lock frequency and allows the Ti-Sapphire laser's operating wavelength to be tuned to wavelengths from 700-1000nm. This tunable wavelength range can be used for two photon excitation (TPE) of a wide range of fluorophores commonly used in conventional fluorescence microscopy.

Fluorescence Lifetime Imaging

The fluorescent relaxation from an excited state is a stochastic process but the lifetime of a fluorescent molecule's excitation has an intrinsic value,

$$\tau = \frac{1}{k_{nr} + \Gamma} \qquad 2$$

where k_{nr} is the non-radiative decay rate and Γ is the radiative decay rate. It follows that the fluorescence intensity from a population of fluorescent molecules, excited at $t = 0$ follows a single exponential decay,

$$I(t) = I_0 e^{-\frac{t}{\tau}} \qquad 3$$

where t is time and I_0 is the fluorescence intensity at $t = 0$, although larger, complex molecules may exhibit multiple lifetimes [4][5]. Unlike fluorescence intensity these intrinsic lifetimes are not dependent on fluorophore concentration or excitation intensity. There are a number of extrinsic factors however which do affect the fluorescence lifetime and changes in its value can be used to quantify environmental features such as oxygen and water concentrations, pH and temperature [8]. Fluorescent lifetimes can also be used to discriminate between spectrally similar fluorophores. Quenching species in the local environment also affect the lifetime by increasing the non-radiative decay rate. An extension of this principle is used in Förster resonance energy transfer (FRET) whereby the excitation is transferred to another fluorescent molecule which is sufficiently close (typically 1-10nm) through non-radiative dipole-dipole coupling. FRET allows the measurement of intermolecular and intramolecular distances including molecular interactions and conformational changes [9][10].

An additional relaxation process which can be experimentally controlled, such as stimulated emission, introduces another rate to Equation 2 which can be manipulated to convey further information and this can be used to reconstruct higher resolution Images [11]. Stimulated emission can occur when a photon, incident on a molecule in an excited state, matches the energy of the transition from the excited state to the ground state. In this case, it is possible that this transition will be induced by the incident photon resulting in the emission of a photon with identical energy to the incident photon. This leads to a shortening of fluorescence lifetime dependent on the intensity of the light used as a "dump". These controlled manipulations of the fluorescence lifetime can be measured and used to

reconstruct higher resolution images. Alternatively a structured, high intensity dump can be used to effectively reduce the possible spatial origin of fluorescence to below the size of a diffraction limited focal spot, again conveying a resolution enhancement.

Anisotropy

Anisotropy defines the extent of polarization of fluorescence emission following excitation by polarized light. Anisotropy, r, is the difference between the fluorescence intensity polarized parallel to the excitation, $I_{\parallel}$, and the fluorescence intensity polarized perpendicularly to the excitation, $I_{\perp}$, normalized by the total fluorescence intensity, $I_T = I_{\parallel} + 2I_{\perp}$, as per equation 4.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad 4$$

The reason fluorescence is anisotropic, is that electronic transitions have moments which lie along specific directions within the fluorescent molecule which in a homogeneous solution will be randomly distributed. Those molecules with their transition moments oriented in the same direction as the electric field of the incident light are preferentially excited and light is emitted with polarization aligned with the transition moment at the time of emission. De-polarization of the fluorescence and a resulting reduction in anisotropy can occur for a number of reasons such as rotational diffusion during the time between excitation and emission. Anisotropy measurements can be used to investigate surface topology, conformational changes in molecules and study molecular dynamics [8].

Absorption and Emission Spectra

Experimental Methods

Absorption and emission spectra of fluorescein and OG488 dyes were taken using an USB4000 Spectrometer (Ocean Optics, UK). The experimental setup for absorption measurements (Figure 3) consist of a xenon white light source which is intensity modulated by a neutral density filter wheel. The white light is focused by a lens onto a dye sample in a 3mm path length quartz cuvette. The transmitted light is refocused into the spectrometer by a second lens. The spectrum of the transmitted light is acquired using the Ocean Optics spectrometer operating software SpectraSuite [12] and the absorbance or optical density, OD_{λ}, at wavelength λ determined by equation 5 from the sample intensity S_{λ}, the background intensity BG_{λ} and the reference intensity R_{λ}.

$$OD_{\lambda} = -\log_{10}\left(\frac{S_{\lambda} - BG_{\lambda}}{R_{\lambda} - BG_{\lambda}}\right) \qquad 5$$

The reference intensity was determined first using a cuvette containing solvent only and the background spectrum was then determined by placing a beam block in the optical path from the source to the detector. All spectra were taken using 200ms time average of the spectrometer's response and an average of 25 spectra was used.

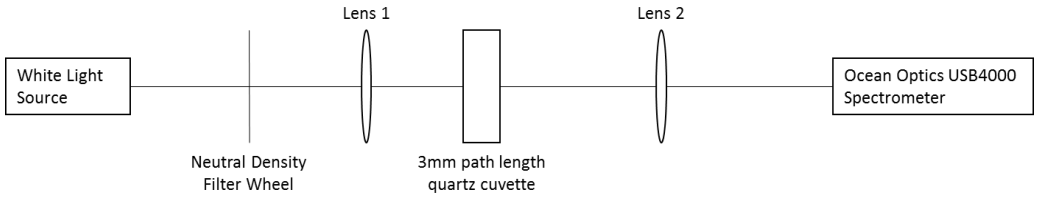

Figure 3: Experimental setup for absorption measurements. A white light source is focused on the centre of a cuvette containing the sample dye. A second lens focuses transmitted light onto a USB4000 spectrometer.

A PicoTA pulsed diode laser (Toptica Photonics AG, Grafelfing, Germany) with a spectral line at 490nm was used to excite fluorescein and OG488 to produce an emission spectrum. The light from this laser was focused into the centre of a 50µl drop of dye on a coverslip using a UPLSAPO 60x 1.2NA water immersion objective (Olympus, Hamburg, Germany) mounted in an IX71 microscope body (Olympus, Hamburg, Germany). The MicroTime 200 time resolved confocal microscope system (PicoQuant, Berlin, Germany) was used to direct the laser into the microscope side port and the USB4000 spectrometer was connected to the exit port of the MicroTime 200 main optical unit (MOU) (Figure 4). The 490nm laser was aligned into the MOU by coupling into an optical fibre connected to the optical fibre input of the MOU. No excitation filter is needed when using a laser for excitation and no detection filters were used in order to preserve as much of the emission spectrum as possible. The major dichroic used was a 51000BS which reflects at wavelengths below 510nm and above 580nm directing the 490nm laser light into the microscope side port and transmitting light at intermediate wavelengths. The pinhole was set to 50µm and Rod 2 was set to the position which directed the emitted light via mirror 5 to the exit port where an optical fibre was coupled to feed light into the spectrometer. The microscope was focused into the sample drop using the image of the back reflection off the top surface of the cover slip, recorded using the diagnostic CCD camera, as a reference point. The pinhole was aligned by maximising the detected signal at the exit port. SpectraSuite was used again to record the response of the spectrometer which was time averaged over 1s and an average of 10 spectra taken.

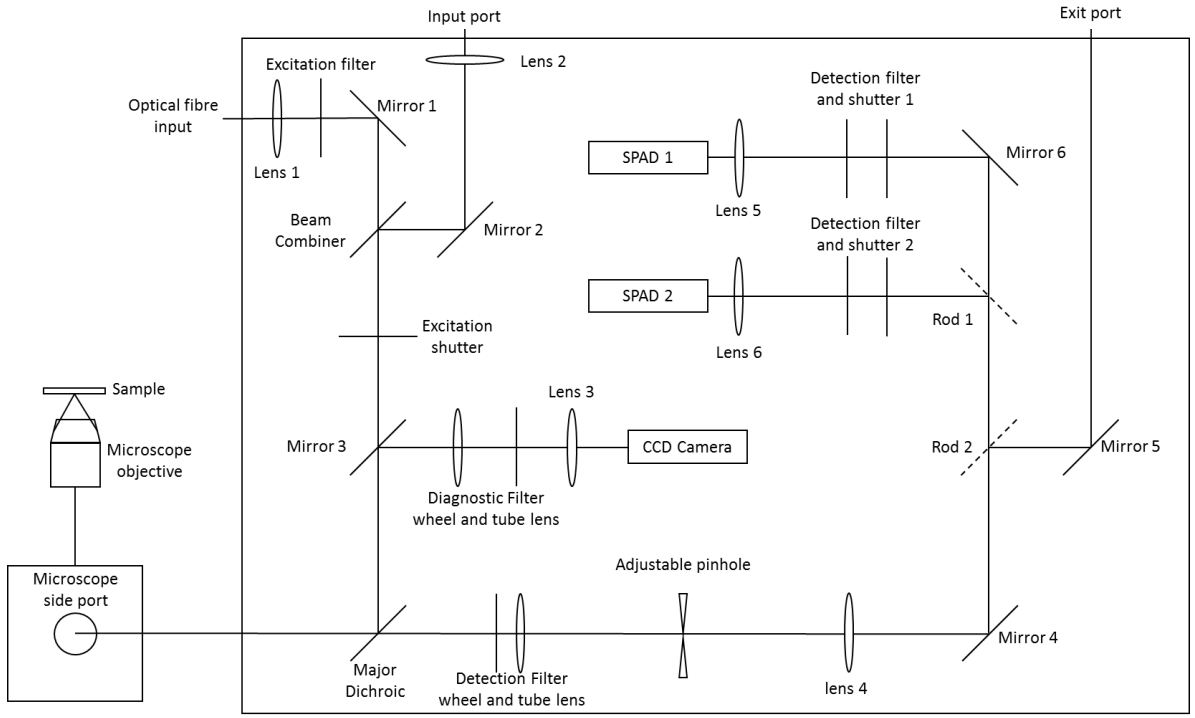

Figure 4: MicroTime 200 time resolved confocal microscope system (PicoQuant, Berlin, Germany) Main Optical Unit. The MOU has an optical fibre input port, a free space input port and a free space exit port. The beam path to and from the microscope side port is also free space. There is a diagnostic beam path to a CCD Camera and the main detector confocal beam path to two single photon avalanche diode (SPAD) detectors. Rod 1 allows the choice of a mirror, 50:50 beam splitter and a cube polariser. Rod 2 allows the choice of additional filters or a mirror diverting the beam to the exit port. For this work no excitation filter or and no detection filters were used on the SPAD beam paths following Rod1.

Results and Discussion

Six approximate concentrations of fluorescein (Sigma Aldrich F2456, Lot #13112JE) and OG488 (Life Technologies 06146, Lot #1396947) were prepared in methanol (MeOH). MeOH was used due to the

poor solubility of OG488 in water. The absorption spectra (Figure 5) of these samples were measured as described. The Beer-Lambert law can be used to derive the relation between optical density, OD, at λ and the concentration, c, giving equation 6,

$$OD_\lambda = \varepsilon_\lambda . c . l \qquad 6$$

where ε_λ is the specific extinction coefficient and l is the path length in cm. It can be seen from the comparison of the predicted OD and the measured OD (Table 1) that the measured values are orders of magnitude different to predicted values. This is due to the limited dynamic range of the USB4000 spectrometer which is not designed to measure ODs above 4. These experiments could be improved by using a sample holder with a shorter path length such that the OD fell below 4 even for the higher concentrations.

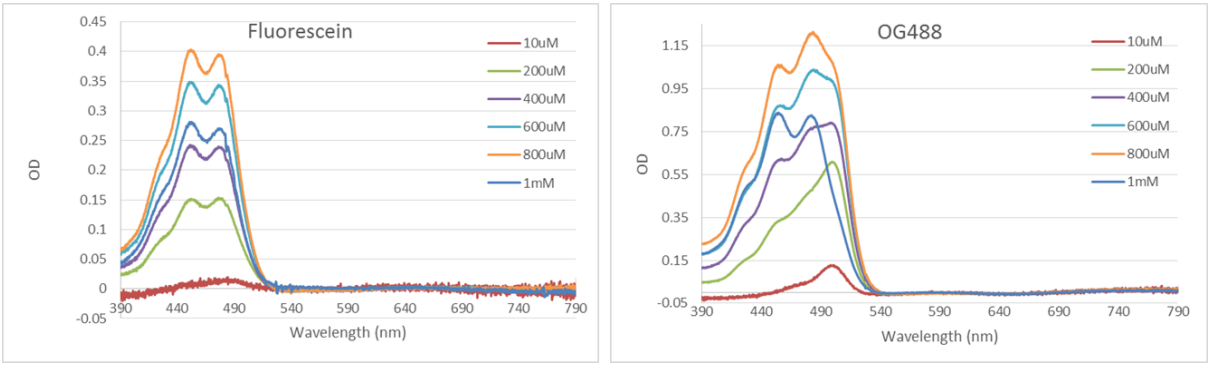

Figure 5: Absorption spectra of fluorescein and OG488 at various concentrations. Adjusted to zero mean OD in the wavelength range 550-750nm.

	Fluorescein - $\varepsilon_{490} = 93000 cm^{-1}mol^{-1}$		OG488 - $\varepsilon_{492} = 85000 cm^{-1}mol^{-1}$	
Concentration (molar)	OD at 490nm	Measured OD at 490nm	OD at 492nm	Measured OD at 492nm
1.00E-03	27.90	0.18 ± 6.50E-03	25.50	0.71 ± 8.70E-03
8.00E-04	22.32	0.27 ± 5.12E-03	20.40	1.16 ± 2.28E-03
6.00E-04	16.74	0.23 ± 3.49E-03	15.30	1.02 ± 1.22E-03
4.00E-04	11.16	0.16 ± 2.77E-03	10.20	0.78 ± 1.30E-03
2.00E-04	5.58	0.11 ± 1.64E-03	5.10	0.54 ± 2.30E-03
1.00E-05	0.28	0.01 ± 1.48E-03	0.26	0.10 ± 3.61E-03

Table 1: Comparison of predicted and measured OD at various concentrations for fluorescein and OG488. Predictions made using Equation 6 with l = 0.3 and ε_λ from product data sheets.

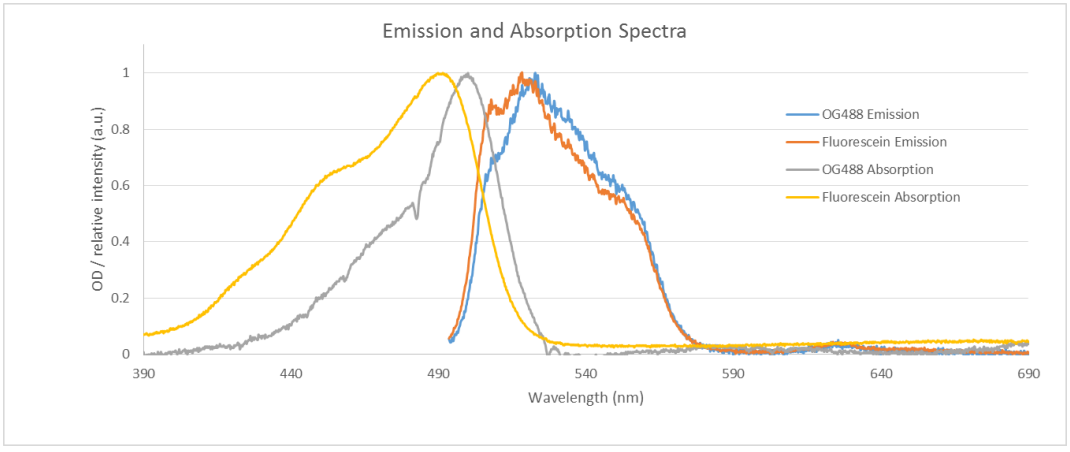

Figure 6: Emission and absorption spectra of fluorescein and OG488 normalised to 1

Absorbance is seen to increase with concentration as expected (Figure 5, Table 1) however this trend is broken by the 1mM measured spectrum. It is thought that measuring the spectrum of the 1mM samples at a different time to the other samples, with a separate reference and background spectrum, combined with exceeding the dynamic range of the spectrometer have all contributed to this anomalous result.

It is not clear, beyond possible experimental error in sample preparation, why the absorbance of fluorescein appears consistently lower than for OG488.

The qualitative conclusion that both fluorescein and OG488 begin to form dimers at concentrations as low as 200µM can be drawn from these experiments however. This is evidenced by the appearance of a second absorption peak at a shorter wavelength than the expected absorption peaks of fluorescein and OG488 between 490 and 500nm.

The emission spectra were acquired as described. To prevent sample evaporation from the coverslip, 10µM samples were prepared by dilution in water of the 1mM samples in MeOH. Absorption spectra were also taken for these samples. Figure 6 shows the normalised emission and absorption spectra of fluorescein and OG488 overlaid. Some curtailing of the emission spectrum has occurred below 510nm and above 580nm due to the use of the 51000BS as the major dichroic. The OG488 absorption spectra is narrower than the fluorescein absorption spectra, possibly making it more amenable for use in applications with multiple dyes where spectral separation is often required.

Time Correlated Single Photon Counting

Experimental Methods

Time correlated single photon counting (TCSPC) experiments were performed using a custom built system (Figure 7). Multiple pinholes are used to ensure the beam is aligned over large distances between the laser and the dark room. A glass coverslip is used to deflect a very small percentage of the beam into the trigering detector for the TCSPC timing electronics. Two neutral density filter wheels allow a large range of intensity modulation of the excitation power. The beam is focused into the sample in a quartz cuvette and scattering of the beam towards the detector is prevented with a number of screens, a beam sink and optical isolation of the detector. Fluorescence emitted by the sample perpendicular to the excitation beam is focused through a pinhole into the optical isolation box containing a microchannel plate photo multiplier tube (MCP) detector (R3809U-01, Hamamatsu Photonics, Japan). A number of filter options are avaliable including a polarising filter whose angle can be controlled from a computer during data acquisition.

The principle of TCSPC is to measure the time between excitation and emission, i.e. the fluorescence lifetime. The excitation pulse from the laser is identified by a constant function discriminator (CFD) which sends a start signal to a time to amplitude converter (TAC). Once the TAC is triggered the voltage is ramped up linearly with time until a stop signal, initiated by the detection of a single photon, is received from the CFD connected to the detector. The voltage on the TAC at the time of a stop signal from the detector is amplified and passed via an analouge to digital converter (ADC) to a PC which logs times in a histogram. The intensity of the excitation is set low enough that fewer than 1% of the excitation pulses result in a fluorescence photon being detected. This reduces the probability of multiple emission events occuring per excitation pulse which would result in a deviation from Poisson statistics.

The 10µM samples prepared by dilution in water of the 1mM samples in MeOH were used again. The polarising filter was set to rotate between parallel and perpendicular positions every 10s and histograms for each position were stored separately. The experiment was performed with fluorescein

and OG488 for both Single photon excitation (SPE) and TPE. SPE was achieved with the PicoTA 490nm laser at a 5MHz repetition rate and power between 17 and 82nW at the sample. The power was measured using an optical power meter (Thor Labs, NJ, USA). TPE was achieved with the MIRA 9000 Ti-Sapphire (Coherent, Santa Clara, CA, USA) mode locked at 800nm. The repetition rate was reduced to 3.7MHz using an acousto-optical pulse picker (Pulse Select, APE, Berlin, Germany) and the power was in the range of 0.73 to 0.83mW at the sample. A BG39 filter was used for TPE to further isolate the detector from excitation scattering and an OG515 was added for the SPE.

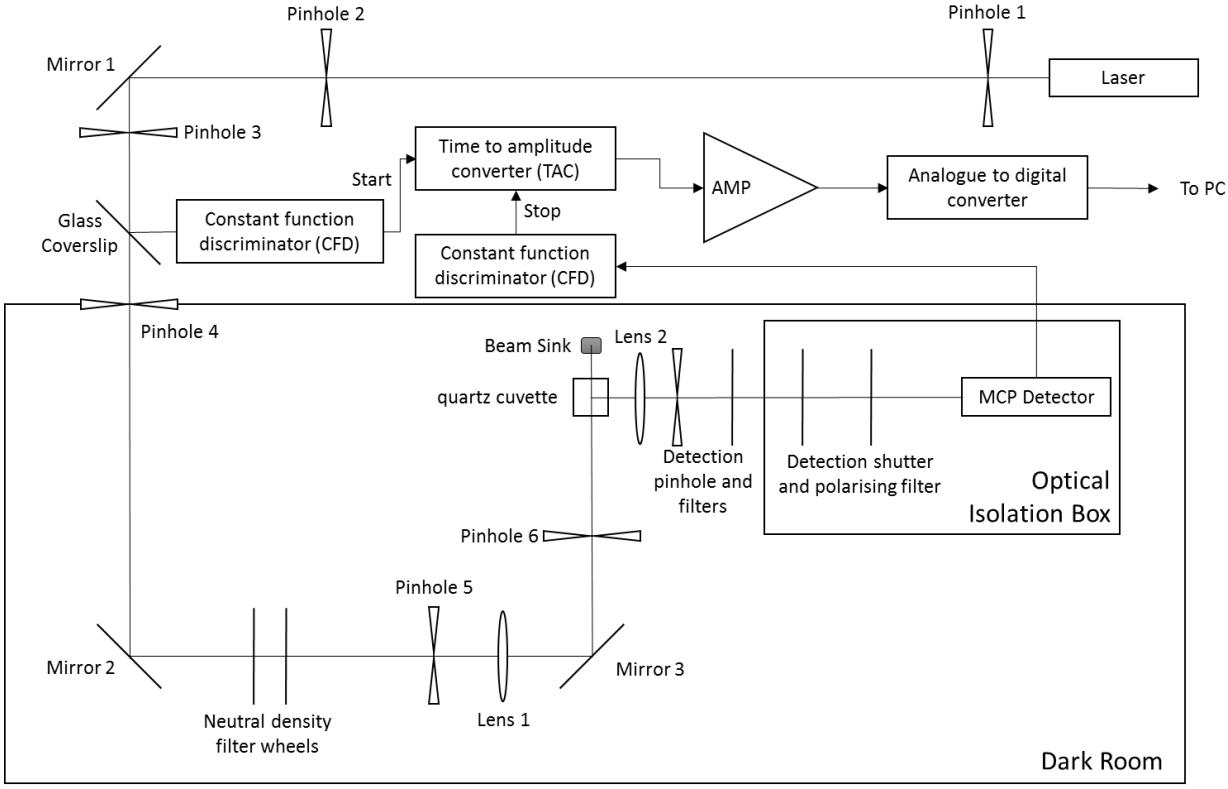

Figure 7: TCSPC beam path and timing electronics. Electronic connections are denoted by arrows.

Results and Discussion

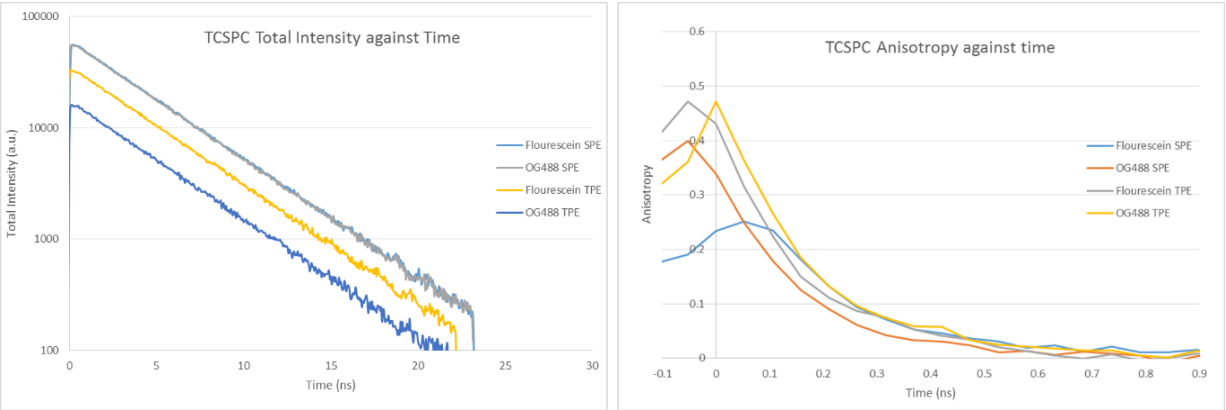

Figure 8: Total intensity and anisotropy data against time from TCSPC experiments using fluorescein and OG488 with single photon excitation (SPE) and two photon excitation (TPE).

Data from TCSPC experiments (Figure 8) was exported to OriginPro (OriginLab, MA, USA) and the fluorescence lifetime determined by nonlinear least squares fitting of a single exponential (Table 2).

Sample	Excitation Method	Lifetime (ns)	Error (ns)	Chi Squared
Fluorescein	Single Photon	4.09E+00	7.43E-03	1.95
OG488	Single Photon	4.06E+00	7.05E-03	2.00
Fluorescein	Two Photon	4.07E+00	8.41E-03	2.18
OG488	Two Photon	4.07E+00	1.16E-02	2.02

Table 2: Fit values for fluorescence lifetime from nonlinear least squares regression performed with OriginPro

The fluorescence lifetimes of fluorescein and OG488 are very similar. OG488 is a derivative of fluorescein and structurally similar so this result is as expected. There is no change in lifetime with TPE as expected for small, simple fluorescent molecules. The Chi squared values of the fits are small and indicated that a single exponential is the correct function to use for fitting. This implies that there is only one excited state which significantly contributes to fluorescence. This is useful for application such as FRET where multiple excitation states, such as found in GFP and mCherry significantly complicate quantitative biological applications.

The anisotropies for fluorescein and OG488 decay to zero in a similar time of the order of 0.5ns (Figure 8). This indicates that the rotational diffusion rate of fluorescein and OG488 in water is very high. This fast decay precluded the accurate fitting of the data due to an insufficient number of data points and make determination of the initial anisotropy difficult. It may be possible to determine this value by deconvolution of the instrument response function from the data, this was not done here. Qualitatively, the initial anisotropy of the TPE is higher than that of the SPE as expected.

CW-Depletion

Experimental Methods

The continuous wave stimulated emission depletion (CW-STED) characteristics of OG488 were investigated using the MicroTime 200 (Figure 4) to perform TCSPC measurements with varying amounts of stimulated emission depletion (STED). Stimulated emission (dumping) was achieved using a Mambo CW diode laser (Cobolt, Solna, Sweden) with a maximum power output of 100mW at a wavelength of 594nm. This laser was coupled into the same optical fiber as the 490nm PicoTA diode laser used for SPE. This inherently leads to a good alignment of the focal spot of the two lasers in the microscope. For the two photon experiments the MIRA 9000 Ti Sapphire laser was used for excitation. The MIRA 9000 was aligned into the MOU through the input port and combined with the Mambo using a beam combiner. Targets on the input port and behind the major dichroic were used to roughly align the two beams. Fine adjustment of the alignment was achieved using the diagnostic CCD camera to record the back reflection from a coverslip and overlaying the foci of the two beams.

50µl drops of the 10µM sample of OG488 previously described and a 10µM sample of the fluorescein in PBS were placed on a coverslip in the IX71 microscope as previously described. A 50µm pinhole was used to achieve confocality, the major dichroic used was the 51000BS which reflects the 800nm and 490nm excitation wavelengths and the 594nm dump wavelength but transmits in the region of the peak fluorescence of fluorescein and OG488. The detection filter wheel was set to an HQ510LP and Rod 2 was set to an FES 550 to filter out both the residual excitation and dump laser light from the detector beam line. Finally Rod 1 was set to a cube polarizer such that vertically polarized emission and horizontally polarized emission was incident on different detectors. Finally, the pinhole, detector lenses and objective are all aligned by maximizing the detected intensity in each of the detectors. The dump beam power was measured using the Thor Labs optical power meter immediately after the major dichroic. The excitation laser repetition rate was 20MHz and count rate was tuned to 1% of the repetition rate. Acquisition time was 30s and TCSPC histograms were produced for varying dump

powers. The fluorescence lifetime was regularly checked with zero dump power for consistency throughout the experiment.

Results and Discussion

The data was acquired using the MicroTime 200 operating software SymPhoTime 64 (PicoQuant, Berlin, Germany) and exported to OriginPro. Results from the TCSPC experiments show that the fluorescence lifetime of fluorescein and OG488 follow a mono exponential decay. The stimulated emission provides a separate path for relaxation of the excited state so bi-exponential functions were fitted to the total fluorescence intensities histograms recorded using nonlinear least squares regression. The fitted lifetimes and the average lifetime of fluorescein for each dump laser power using SPE are plotted in Figure 9, error bars represent fitting errors and standard deviation in power measurements. The results show one lifetime which remains close to its initial value and a second lifetime which is significantly reduced as the dump power is increased. The same result is seen for OG488 (individual lifetime data not shown, average lifetime data Figure 10).

The fractional depletion (Fd) can be calculated from the fractional difference in the un-dumped fluorescence lifetime and the average dumped fluorescence lifetime (Equation 7).

$$Fd = \frac{t_{un-dumped} - |t_{dumped}|}{t_{un-dumped}} \qquad 7$$

Both the average fluorescence lifetime and the fractional depletion of OG488 (Figure 10) are very similar for SPE and TPE. The plateau of these values with increasing dump power does appear to occur at slightly higher power for the TPE. High fractional depletions are necessary for applications such as STED which relies on complete de-excitation in the peripheries of the focal region to achieve good resolution improvements. However, this turnover in the fractional depletion is observed for all fluorescent dyes previously studied in solution using this method (unpublished results) and does not imply OG488 is unsuitable for STED applications. OG488 has been successfully used as a STED probe previously.

The difference in anisotropy between the un-dumped and dumped data, δR, for TPE of OG488 (Figure 11) is zero for all times after excitation and all dump powers. This shows STED has no effect on the anisotropy of OG488. This was also seen for fluorescein and for SPE (data not shown).

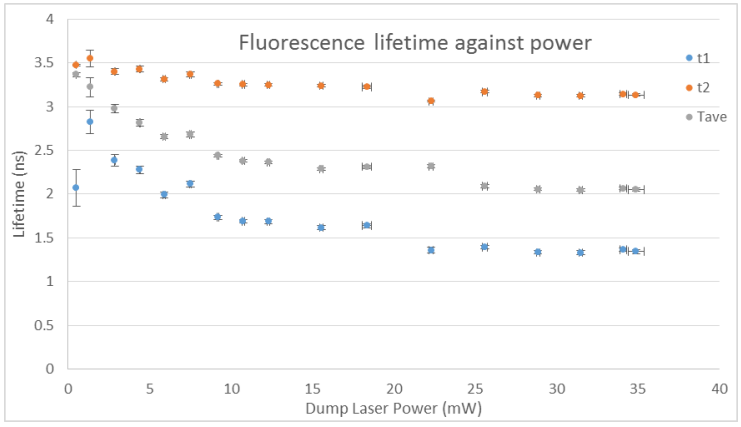

Figure 9: Fluorescence lifetime against power for fluorescein using single photon excitation and varying dump laser powers. Error bars show fitting errors of the nonlinear least squares fit performed in OriginPro.

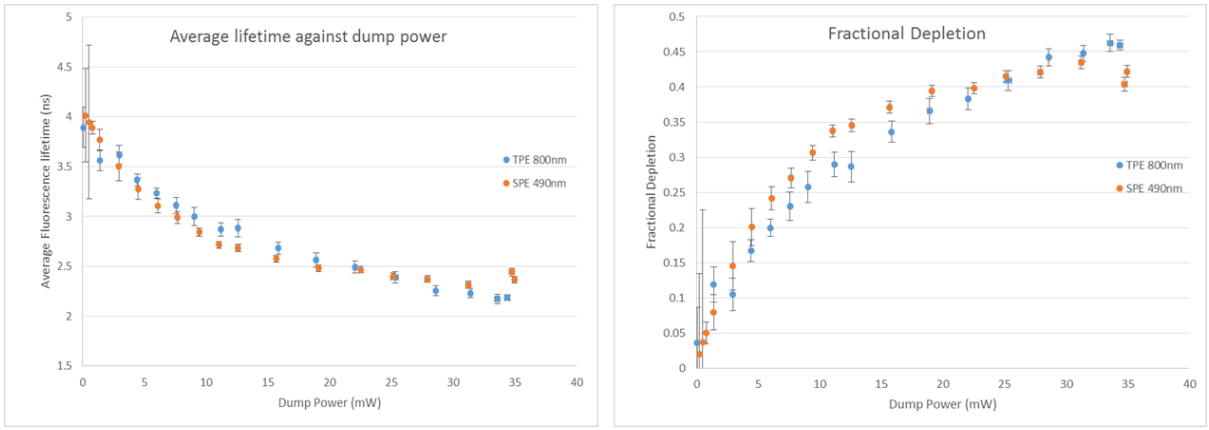

Figure 10: Comparison of single photon excitation and two photon excitation of OG488 with respect to: Left, Average lifetime and right, fractional depletion. Error bars are calculated using the error in the component lifetimes from the fit values calculated in OriginPro.

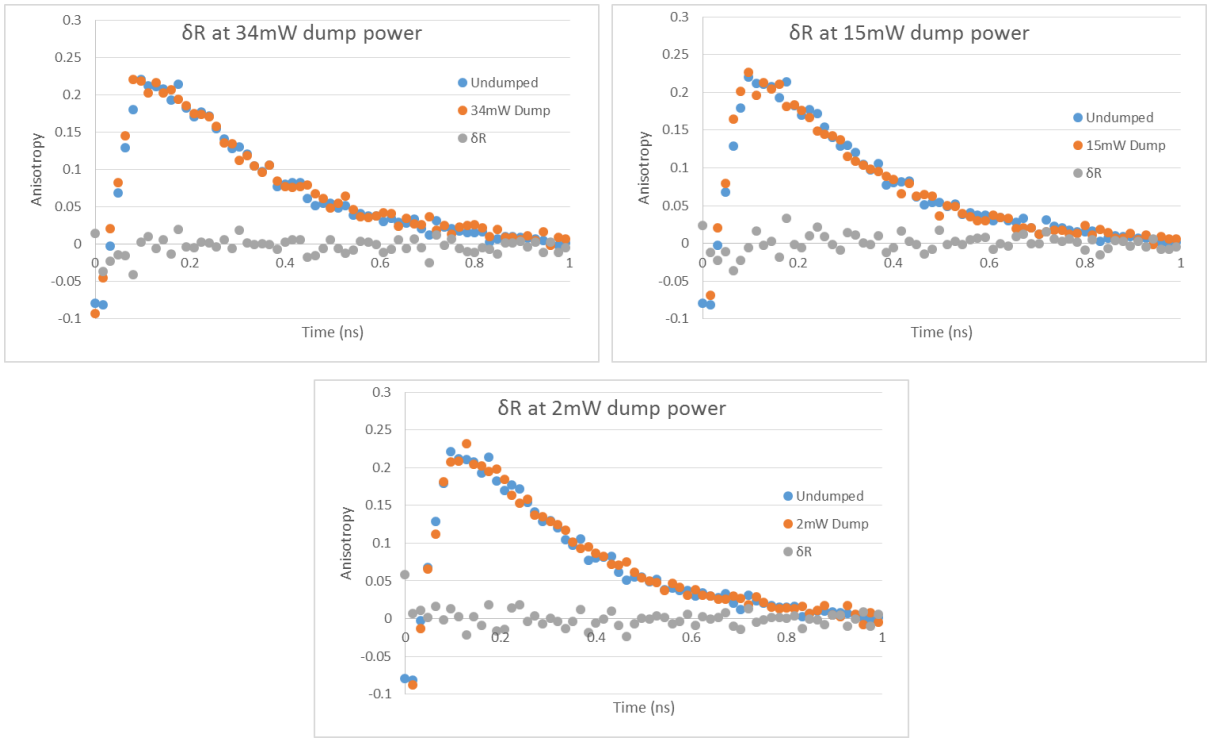

Figure 11: Change in anisotropy at varying dump powers for two photon excitation of OG488

Quantum Yield

Experimental Methods

Quantum yield is defined as

$$\Phi = \frac{Photons\ Emitted}{Photons\ Absorbed} \qquad 8$$

and was measured for OG488 using both the absorption (Figure 3) and TCSPC (Figure 7) experimental setups following a method published online by Horiba Scientific [13]. This method relies on the comparison of absorbance and fluorescence intensity of a reference sample with a known quantum yield to that of the test sample with unknown quantum yield. From the linear relation between fluorescence intensity and absorbance, it can be shown that

$$\Phi_{test} = \Phi_{Ref} \frac{m_1}{m_2} \quad (9)$$

where m_1 and m_2 are the gradients of the fluorescence intensity against absorbance for the test sample and the reference sample respectively. These gradients can be determined experimentally by measuring the absorbance and fluorescence at varying concentrations. To remain within the linear regime reabsorption effects must be avoided by ensuring the OD is of the order of 0.03 or less for a 3mm path length [13]. Taking into account the sensitivity of the spectrometer this results in a usable concentration range of 0.1 to 1µM. 6 concentrations of fluorescein (the reference sample) and OG488 (the test sample) were prepared in this concentration range by appropriate dilution in water from the 1mM samples in MeOH.

The polarizer in the TCSPC experimental setup was set to the magic angle (54.7°) to remove de-polarization effects and the MCP detector was used to measure the total fluorescence intensity of each of these samples. The PicoTA 490nm laser was used to excite the samples. The OD at 490nm was calculated from the absorption spectrum acquired as previously described. The laser power was measured at the sample, at the time of the intensity measurements and all experiments were done in the dark.

Results and Discussion

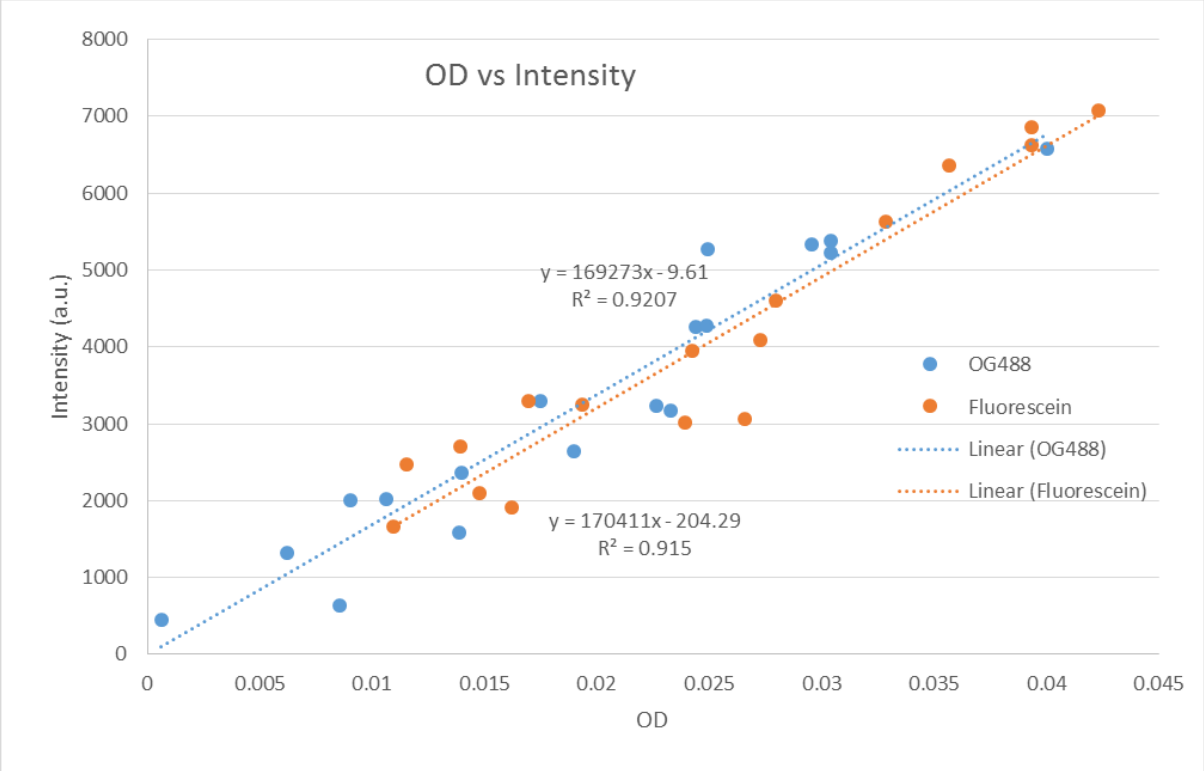

Figure 12: Combined data from three quantum yield experiments

The experiment was repeated three times with on sample excitation powers of 4nW, 14nW and 20nW. The data were corrected for excitation power and plotted in OriginPro, a linear fit performed (Figure 12) and quantum yields calculated from the value for fluorescein of 0.92±0.02 [14]. The value determined was 0.91±0.05.

Two Photon Cross Section

Experimental Methods

Two photon cross sections can be determined for a sample of known quantum yield by measuring the time-averaged total fluorescence intensity upon TPE with a pulsed laser source [15]. Accurate measurement of the temporal profile of the laser pulses in the sample volume can be avoided by comparing the fluorescence intensity of a test sample to that of a reference sample, with a known two photon cross section, σ_{2P-Ref} (Equation 10).

$$\sigma_{2P-test} = \sigma_{2P-Ref} \frac{\Phi_{Ref} n_{Ref} C_{Ref} \varepsilon_{Ref}}{\Phi_{test} n_{test} C_{test} \varepsilon_{test}} \frac{\langle I_{Total}(t) \rangle_{test}}{\langle I_{Total}(t) \rangle_{Ref}} \quad 10$$

where Φ is the quantum yield, n is the refractive index C is the concentration and $\langle I_{Total}(t) \rangle$ is the time averaged total intensity. ε is a correction factor for the part of the emission spectrum which is not detected due to the filters used and the detector efficiency, calculated as

$$\varepsilon = \frac{\int_{\lambda_{min}}^{\lambda_{max}} E(\lambda) F(\lambda) G(\lambda) d\lambda}{\int_{\lambda_{max}}^{\lambda_{min}} E(\lambda) d\lambda} \quad 11$$

where $E(\lambda)$ is the emission spectrum of the fluorophore and $F(\lambda)$ and $G(\lambda)$ are the transmission efficiencies of the filter and detector respectively.

The TCSPC experimental setup was used with polarizer set to 54.7°, a BG39 filter was used and the MCP was used to measure fluorescence intensity. The samples were two photon excited using the Mira 9000 tuned at wavelengths from 730nm to 890 nm in 10nm steps. The emission spectrum and quantum yield of OG488 were determined above. The two photon cross section spectrum and quantum yield of rhodamine B (Rod B) is known so was used as the reference. ε was calculated for Rod B and OG488 using transmission efficiencies previously determined in the lab. 10µM concentrations, confirmed by an absorbance measurement, were used.

Results and Discussion

Figure 13: Two photon cross section of OG488, including the two photon cross section of Rod B used as a reference.

The OG488 has a similar two photon cross section to Rob B, in the range measured (Figure 13) and has a peak between 780nm and 800nm. Ti-sapphire lasers, commonly used in two photon microscopy, are most efficient at 800nm so OG488 is an appropriate choice of fluorescent probe for two photon microscopy.

Concluding Remarks

Knowledge of the photophysical characteristics of fluorescent molecules can have a significant impact on quantitative biological research techniques. With new molecular probes, such as OG488, becoming available their characterisation provides a greater choice of probes for specific applications.

Here, the absorption and emission spectra of OG488 have been measured and it is shown that dimerization of OG488 can occur at high concentrations changing the absorption spectrum. This is significant if OG488 is used in experiments where local concentrations of the OG488 rise above 10s of µM.

The fluorescence lifetime and anisotropy of OG488 have been calculated. Knowledge of these intrinsic values allows the use of OG488 as a probe to investigate micro environments and the molecular dynamics molecules it can be fused with.

The Fractional depletion of OG488 in solution under stimulated emission has been calculated for a range of dump powers. The maximum Fd achieved was 50%. This is an important consideration for the STED microscopy field which relies on 100% depletion of fluorescent dyes to convey reliable improvements in resolution.

The previously unpublished quantum yield of OG488 has been measured as 0.91 ± 0.05. This is an important value for a number of quantitative methods such as FRET based distance measurements. OG488 may become an important FRET probe since its fluorescence lifetime exhibits a mono-exponential decay making quantitative predictions more straightforward than probes such as GFP.

Finally, a two photon cross section spectrum was measured indicating that OG488 would be a very suitable TPE probe.

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