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### Two-Photon Induced Fluorescence

Two-photon fluorescence (TPF) is a nonlinear spectroscopic process involving the simultaneous absorption of two photons. Maria Goppert-Mayer first predicted two-photon absorption (TPA) in 1931 but it was not until the 1960's, with the development of lasers, that Kaiser and Garret first observed it experimentally.<sup>1,2</sup> TPF is complementary to single-photon fluorescence (SPF) in that it has different selection rules and is able to examine excited states that are not accessible by SPF. Some of the advantages of using TPF over SPF are a large signal-to-noise ratio, small probe volumes, three-dimensional imaging capability, limited photodamage, and increased penetration into absorbing samples. TPF has many applications including chromatographic detection, two-photon excitation fluorescence microscopy, and photon scanning tunneling fluorescence microscopy.

When light interacts with a molecule it results in the polarization of the electron cloud by the electric field component of the light. The molecule can absorb one or more photons and the resulting polarization can be described by equation 1.<sup>3-5</sup>

$$(1) \quad \mathbf{P}(\mathbf{r},t) = \chi^{(1)} \mathbf{E}_a(\mathbf{r},t) + \chi^{(2)} \mathbf{E}_a(\mathbf{r},t) \mathbf{E}_b(\mathbf{r},t) + \chi^{(3)} \mathbf{E}_a(\mathbf{r},t) \mathbf{E}_b(\mathbf{r},t) \mathbf{E}_c(\mathbf{r},t)$$

a, b, c: three different photons

$\mathbf{P}(\mathbf{r},t)$ : polarization of the molecule

$\chi^{(1)}, \chi^{(2)}, \chi^{(3)}$ : 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> – order susceptibilities respectively

$\mathbf{E}_a, \mathbf{E}_b, \mathbf{E}_c$ : electric field of the excitation light

The susceptibilities are tensors that relate the electric field components (three-dimensional) of the absorbed light to the resulting polarization (one-dimensional) of the molecule. With low field strengths  $\chi^{(1)}$ , a second-rank tensor, dominates to give only single-photon absorption with linear polarization of the medium. At higher electric field strengths, higher order susceptibilities come into play resulting in multiphoton absorption (MPA) with nonlinear polarization.  $\chi^{(2)}$  is a third-rank tensor that describes second order processes such as TPA. The second-order susceptibility is surface specific and does not occur in molecules that possess centers of symmetry either on the microscopic (molecular) or macroscopic (bulk) level.<sup>4,5</sup> It is common practice to induce asymmetry by placing the molecules at an interface or on a surface (e.g., self-assembled monolayers).<sup>6</sup>  $\chi^{(3)}$  is a fourth-rank tensor that

describes third order processes such as three-photon absorption. Third order processes can occur in any medium regardless of symmetry.<sup>4</sup> The probability that more than one photon can be absorbed simultaneously scales with intensity raised to the nth power where n is equal to the number of photons absorbed.<sup>7</sup>

Multiphoton fluorescence involves the absorption of multiple photons to an excited electronic state followed by the relaxation of the molecule to the ground electronic state through the emission of a single photon. The wavelength of the emitted photon is approximately equal to the excitation wavelength divided by the number of photons absorbed. Typically an IR laser is used to excite UV absorbing chromophores. The energy absorbed by the molecule can be calculated by a modified version of Planck's Law ( $\Delta E = hv_1 + hv_2$ ).<sup>8</sup> One selection rule for SPF is that the parity must change in order for a change in transition moment to occur. SPF commonly promotes transitions from gerade (even, g) to ungerade (odd, u) states. TPF can be thought of as two consecutive single-photon excitations. This results in TPF spectra that are complementary to SPF. Two-photon excitation normally examines transitions from gerade ground states to gerade excited states with ungerade intermediate states.<sup>3</sup> It is important to note that although transitions may be allowed by the parity selection rule, they may be prohibited by other symmetry rules. The excitation wavelength doesn't affect the fluorescence spectrum as long as the same electronic transition is examined. This can be attributed to the dissipation of the excess energy through radiationless decay from a vibrationally excited state to the vibrational ground state.<sup>2,9</sup> A unique aspect of multi-photon fluorescence compared to SPF is that the polarization information of the molecule is retained and it can be used to identify symmetry properties of the molecule.<sup>3</sup>

In MPA, the absorption rate ( $Ex(t)$ ) of the molecule can be determined using equation 2.<sup>10,11</sup>

$$(2) \quad Ex(t) = \delta I^n$$

$\delta$ : absorption cross section  
 $I$ : intensity of the excitation source  
 $n$ : number of photons absorbed

The maximum absorption cross section found in equation 2 can be estimated using equation 3.<sup>10,11</sup>

$$(3) \quad \delta_{\max} = 10^{-15} \text{ cm}^{2n} * [10^{-23} \text{ s/photon}]^{n-1}$$

The absorption cross section for TPF is approximately  $10^{-48} \text{ cm}^4/\text{photon}$ . The Goppert-Mayer unit ( $1\text{GM} = 10^{-50} \text{ cm}^4/\text{photon}$ ) is a shorthand notation for absorption cross sections.<sup>10,11</sup> A molecule that has become electronically

excited through the absorption of a photon or multiple photons can undergo several different processes. The excess energy can be dissipated through internal conversion, also known as radiationless decay (lifetimes of  $10^{-12} - 10^{-15}$  s). The energy can also be released through the emission of a photon by fluorescence ( $10^{-8}$  s) or phosphorescence ( $10^{-3}$  s or longer). The factors affecting fluorescence are solvent relaxation, fluorescence quenching and excited state reactions.

There are several problems affecting fluorescence spectroscopy. One problem in fluorescence detection is interference from other molecules in the sample that absorb the same wavelength as the fluorophore. This is known as the inner-filter effect. TPF does not experience this problem since the emission wavelength is far removed from the excitation wavelength.<sup>3</sup> Another problem faced in fluorescence is the absorption of the emitted fluorescence by other molecules in the sample. TPF can get around this problem by focusing the laser beam near the face of the sample cuvette thereby decreasing the distance the emitted photons have to travel in order to reach the detector.<sup>3</sup> An advantage that TPF has over SPF has to do with the nature of the IR excitation source. Since the wavelength of the excitation source is red shifted by several hundred nanometers from the fluorescence (UV region), scattered light, both Raman and Rayleigh, can be easily filtered from the signal. Since longer wavelengths are used, less scattering of the source occurs resulting in deeper penetration of the IR light into the sample. Another advantage of TPF over SPF is that IR lasers are less costly and complex than UV lasers and their associated optics.<sup>12</sup> One requirement of TPF is that a high intensity laser must be used. This can be achieved by focusing the laser beam to its diffraction-limited radius with TFA only occurring near the focal point.<sup>7</sup> This gives TPF the advantage of working with small sample volumes, and limits photobleaching and photodamage to areas directly around the focal point.<sup>13,14</sup>

The absorption rate for two-photon excitation depends on both the spatial and temporal coherence of the laser.<sup>2</sup> TPF can be obtained using either continuous wave (CW) or pulsed lasers, but CW lasers require  $10^2 - 10^3$  times more average power than pulsed lasers. Pulsed lasers with high repetition rates and low duty cycles are the most effective by providing high peak intensities, low average intensity, along with rapid cycling of the fluorophore resulting in multiple excitations.<sup>12</sup>

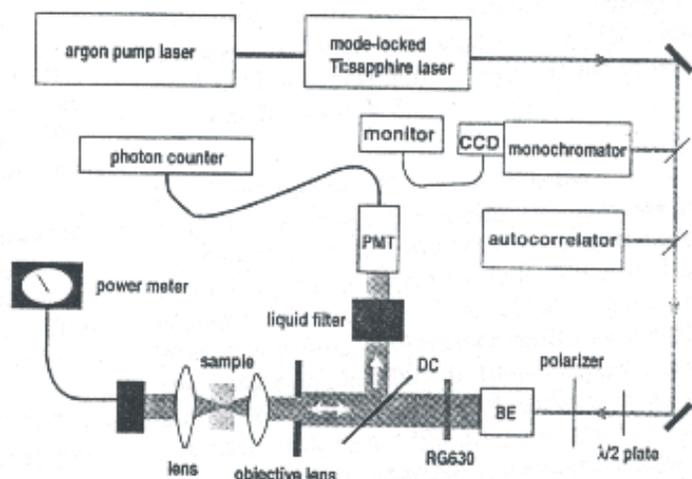


Figure #1: TPF Detection

intensity on the sample is controlled using the combination of a half-wave ( $\lambda/2$ ) plate and a Glan-Thompson polarizer. A 5x beam expander (BE) is used to increase the diameter of the laser beam to fully illuminate the back of the microscope objective. This microscope objective serves the dual purpose of both focusing the incoming laser beam onto the sample and collecting the resulting fluorescence. Scattered light is filtered from the fluorescence using a two-fold approach. It involves using a long-pass dichroic (DC) mirror that reflects the fluorescence onto the detector while transmitting the scattered light. The fluorescence must then pass through a liquid barrier-filter filled with a 1M solution of  $\text{CuSO}_4$  that absorbs the remaining scattered light from the laser before being detected by the photomultiplier tube.<sup>2</sup>

TPF is a useful nonlinear optical technique that can be used in conjunction with both SPF and higher order techniques to more completely characterize the molecular transitions. Some advantages of TPF are the higher signal-to-noise, spatial localization of photobleaching, smaller sample volumes, increase penetration depth into turbid samples, and the use of IR lasers instead of UV lasers. Some disadvantages of TPF include a small absorption cross section resulting in decreased signal and the need for high intensity lasers to get adequate excitation of the sample. TPF has been used as a detector for chromatographic techniques such as capillary electrophoresis.<sup>12</sup> It has also been applied to imaging techniques such as laser scanning microscopy (LSM)<sup>15</sup>, near- and far- field fluorescence microscopy<sup>16</sup>, and photon scanning tunneling microscopy (PSTM).<sup>17</sup>

The setup for TPF is illustrated in Figure #1. In this setup an argon laser is used to pump a mode-locked Ti:sapphire laser. The pulse width of the laser is monitored using an intensity autocorrelator and the spectrum is monitored using a CCD camera. This setup is has a spectral range of 690 to 1050 nm requiring specially coated intracavity mirrors. The illumination

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