Two-Photon Fluorescence Excitation by Picosecond Diode Lasers

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Abstract: Picosecond diode lasers can be used for two-photon excitation of typical fluorophores used in biomedical spectroscopy. A 10 mW, 785 nm, 300 ps pulse width, 50 MHz repetition rate laser was focused into a dye cell by an NA = 0.4 microscope lens. The fluorescence was detected by a cooled GaAs-cathode PMT and recorded by time-correlated single photon counting. A count rate of 4500 photons per second was obtained from a 10^{-3} mol/l Rhodamine 6G solution.

Two-Photon Excitation

Two-photon excitation is based on the simultaneous absorption of two photons of the excitation light. The excitation wavelength is twice the absorption wavelength of the molecules to be excited. The excitation efficiency increases with the square of the excitation power density. Therefore, pulsed excitation with high peak power, i.e. short pulses, and focusing into a small spot of the sample are required to obtain a noticeable efficiency.

Two-photon excitation has a number of remarkable features. The most obvious one is that a noticeable level of excitation is obtained only in the waist of a focused laser beam. This gives two-photon excitation an inherent depth resolution [2]. Moreover, the excitation is on the long-wavelength side of the absorption band. The one-photon absorption coefficient of the sample at this wavelength is usually very low. Therefore, the excitation light easily penetrates into deep layers of a sample [6, 9]. Excitation of deep sample layers is further improved by the reduced scattering coefficient at long wavelengths. Two-photon excitation has therefore become a standard technique of laser scanning microscopy [5, 10]. It is also used for single-molecule experiments by fluorescence correlation spectroscopy [1] and burst-integrated lifetime techniques [3]. These applications make use of the fact that the ratio of the cross sections of the absorption of investigated molecules and the background effects is usually much higher than for one-photon excitation. Two-photon excitation may also be used in standard fluorescence lifetime experiments to record fluorescence in the presence of strong non-fluorescent absorbers, for fluorophores of low stokes shift, or to improve the selectivity of the excitation of specific electronic states [7, 8, 11, 12, 13]. Fluorescence anisotropy decay measurements benefit from the higher initial anisotropy [13, 14].

Two-photon laser scanning microscopes and two-photon single-molecule experiments use femtosecond Ti:Sapphire lasers. The typical power at the sample is between about 10 mW and several 100 mW. Two-photon fluorescence excitation with a picosecond diode laser cannot be expected to work with the same efficiency. The average intensity is only a few mW, and the pulse duration is of the order of 100 ps. The relative excitation efficiency of different lasers can be estimated as follows:

The peak power of the laser is approximately

$$P_{peak} = \frac{T_{per}}{T_{pw}}$$

(P_{peak} = peak power, P_{av} = average power, T_{per} = laser pulse period, T_{pw} = laser pulse width)

The excitation efficiency, E_{ex}, increases with the duration of the excitation pulse, T_{pw}, and with the square of the peak power, P_{peak}.

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$$E_{ex} = k \cdot P_{peak}^2 \cdot T_{pw}$$

or

$$E_{ex} = k \cdot P_{av}^2 \frac{T_{per}^2}{T_{pw}}$$

The factor *k* depends on the two-photon absorption cross section of the dye at the laser wavelength, on the pulse shape, and on the spatial energy distribution in the focus. Because the effective excitation depends on the reciprocal pulse width - not on its square - the prospects to detect diode-laser excited two-photon fluorescence are not too bad. It has indeed been demonstrated that two-photon excitation at a useful level can even be achieved with CW lasers [4].

Experiment Setup

Our experiment to demonstrate two-photon excitation by diode lasers is shown in Fig. 1. The laser (BHLP-700, Becker & Hickl) emits pulses at a repetition rate of 50 MHz and a wavelength of 785 nm. The average power of the laser was adjusted to approximately 10 mW. The pulse width was determined by TCSPC and was about 300 ps FWHM.

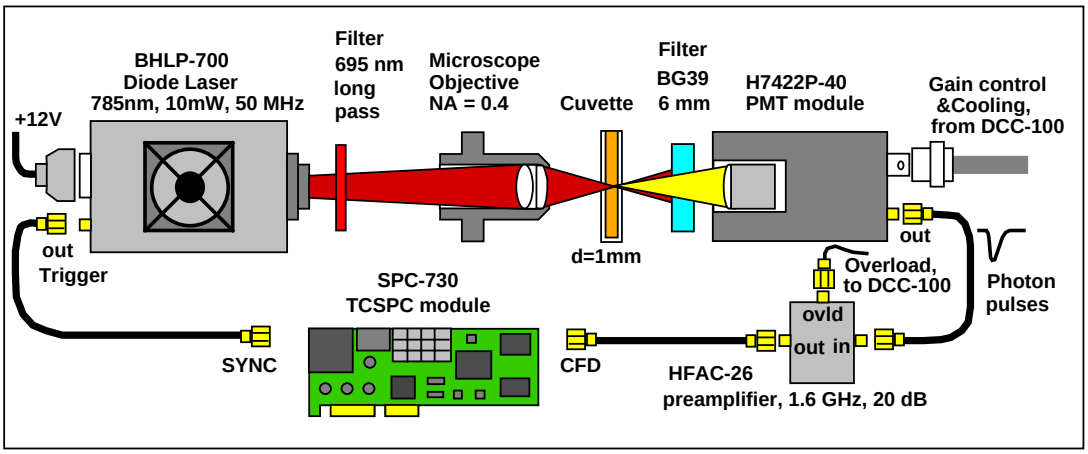

Fig. 1: Experimental setup

The laser beam was focused into a 1-mm sample cell by a simple microscope objective lens (NA = 0.4). The relatively low NA was used to obtain a convenient working distance to the sample cuvette. The used microscope lens was corrected for microscopes without a tube lens. The laser collimator was therefore adjusted to get a slightly divergent beam. This allowed the objective lens to be operated close to the beam geometry for which it was designed and to exploit its full aperture.

Diode lasers emit a noticeable amount of light at wavelengths different from the nominal emission wavelength. To block emission below 650 nm a 695 nm long-pass filter glass was put into the excitation beam.

The fluorescence light was detected from the back of the cuvette. 6 mm of Schott BG39 filter glass was used to block the laser light from the detector. This somewhat unusual geometry has the benefit that, by shifting the sample longitudinally, the illuminated area can be changed without changing the total incident power. Shifting the sample out of focus provides and easy means to verify that two-photon excitation is on work.

The detector was a H7422P-40 cooled photomultiplier module (Hamamatsu). The module was controlled via a DCC-100 detector controller (Becker & Hickl). The single-photon pulses were

amplified by a 20db, 1.6 GHz preamplifier and fed into the start input of an SPC-730 TCSPC module (Becker & Hickl). The trigger output pulses from the laser were used as stop pulses.

Results

The feasibility of two-photon excitation was tested with 10^{-3} mol/l and 10^{-4} mol/l solutions of Rhodamine 6G in ethanol and Fluorescein-Na in water. The results are shown in Fig. 2.

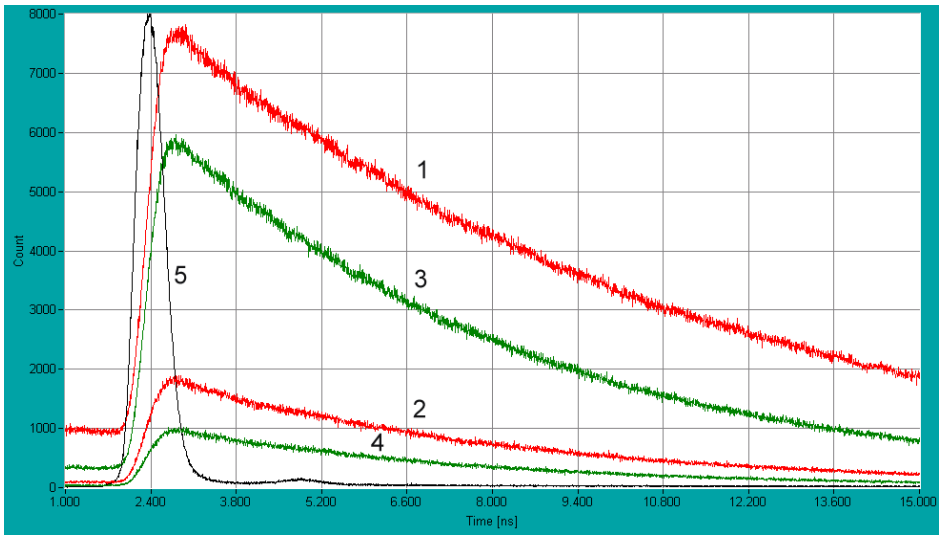

Fig. 2: Fluorescence decay curves obtained be two-photon excitation
1) Rhodamine 6G, 10^{-3} mol/l in ethanol, 2) Rhodamine 6G 10^{-4} mol/l in ethanol,
3) Fluorescein-Na 10^{-3} mol/l in pH 7.4 buffer, 4) Fluorescein-Na 10^{-4} mol/l in pH 7.4 buffer.
5) Laser pulse

Curves 1, 2, 3, and 4 are the recorded decay functions of Rhodamine 6G, 10^{-3} mol/l, Rhodamine 6G 10^{-4} mol/l, Fluorescein 10^{-3} mol/l and Fluorescein 10^{-4} mol/l. The count rates were 4500 s^{-1}, 900 s^{-1}, 3000 s^{-1} and 400 s^{-1}, respectively. The overall acquisition time was 3000 s each. The fluorescence lifetimes are 5.4 ns and 7.9 ns for the 10^{-4} mol/l and 10^{-3} mol/l Rhodamine 6G solutions, and 4.7 ns and 5.6 ns for the 10^{-4} mol/l and 10^{-3} mol/l Fluorescein solutions. The increase of the lifetime with the concentration can be explained by re-absorption. Re-absorption increases with the optical thickness of sample. It more noticeable for two-photon than for one-photon excitation, because locations deeper in the sample are be excited.

Curve 5 is the laser pulse, detected through ND filters without the BG 39 blocking filter. The laser pulse was recorded to get a time reference as to where the fluorescence is to be expected. Please note that the recorded laser pulse is not exactly the profile of the instrument response function (IRF). The two-photon effect is proportional to the square of the power, therefore the two-photon IRF is different from the recorded laser pulse.

Discussion

The detected photon rates of 400 to 4500 photons per second are relatively low. Therefore the results may not appear very promising at first glance. It should, however, be taken into regard that the used setup was not optimised for maximum excitation and detection efficiency. In particular, the efficiency can be improved by

- Using a high-NA lens, e.g. an immersion lens with NA = 1.3. This should improve the excitation efficiency by a factor of 10.

- Beam shaping of the excitation beam. The beam from a laser diode has a noticeable astigmatism and is not focused into a diffraction limited spot.
- Using a dielectric blocking with a steeper transition characteristic. Especially for the Rhodamine 6G, the used BG39 filter glass blocks more than 80 % of the fluorescence.
- Matching of the laser wavelength and the absorption maximum of the fluorophores. The used wavelength of 785 nm is considerably shorter than the wavelength of the two-photon absorption maxima.
- Collection of the fluorescence photons through the microscope objective and diverting the fluorescence signal by a dichroic mirror. With a high NA objective, this increases the NA of the detection light path. Compared to the test setup (an 8 mm photocathode in a distance of 20 mm) an increase in detection efficiency by a factor of 5 to 10 can be expected.

All in all, an improvement in the detected count rate by a factor of 10^3 appears feasible. If this is correct, diode laser excited two-photon fluorescence can be expected to be detectable from a large number of marker dyes in biologically relevant concentrations.

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