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Two-photon excited fluorescence microscopy combined with spectral and time-resolved measurements for fluorophore identification

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1. Abstract

Two-photon excited fluorescence microscopy was used to study unstained tissue and paper samples. As an excitation source a mode-locked Ti:Sapphire laser was utilised. In the experiments we used a conventional fluorescence microscope with a scanning board. The incoming laser pulses were focused onto the sample and the epifluorescence observed. In the spectroscopic measurements the fluorescence light was projected either on the slit of an polychromator with a CCD camera or, in some experiments, on a streak camera connected to the polychromator. The signal was then detected by a 2D-CCD camera. Fluorescence images were scanned by recording the fluorescence light pixel by pixel with a photomultiplier tube. Signal filtering and image processing were performed on a personal computer. Tissue samples from animals treated with photodynamic therapy were examined. The tissue contained protoporphyrin IX as a photosensitiser.

2. Introduction

2.1. Two-photon excited fluorescence microscopy

Fluorescence is a powerful technique, that can provide information about the fluorophore composition of examined samples. Fluorescence microscopy is widely used to study biological specimens. Normally staining procedures are adopted in such studies for identifying and marking molecular classes, but also endogenous fluorophores are investigated. Characteristic fluorescence spectra can be obtained for different tissue fluorophores. In order to perform spectroscopic measurements in small samples a confocal set-up of a microscope can be used. Due to the confocal geometry of such a microscope, fluorescence light from out-of-focus regions can be rejected, enabling spectroscopic point monitoring as well as three-dimensional image scanning.

An alternative approach to confocal scanning laser microscopy (CSLM) is two-photon excited fluorescence microscopy (TEFM)[1,2], a new fluorescence microscopy method under development. This method has several similarities with confocal microscopy and has also some unique features of interest for certain applications. The method is based on a technique, in which extremely short laser pulses with high peak-power are focused to a diffraction-limited spot with a high numerical aperture lens. A laser wavelength corresponding to the need of two photons to excite the sample molecules must be used (usually light in the NIR region, where one photon does not provide enough energy to excite any molecules to an excited electronic state). The excitation probability thus depends on the square of the intensity and only in the focus the intensity is high enough to produce a detectable fluorescence signal.

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This excitation method therefore intrinsically confines the volume, from which a signal is generated, to the very focal volume. Only extremely weak signals can be observed from out-of-focus areas. An important advantage for 3D imaging is, that not only the fluorescence, but also the bleaching (at least theoretically) is confined to the focal volume, making it possible to measure unbleached signals for each depth, independent on previous measurements at other depths. The chromatic lens aberrations can be less severe for red light than for light in the UV-region, as it is used for one-photon excitation. Also, since no pinhole is required in front of the detector, the instrument can be easier adjusted and is more robust than a normal confocal microscope.

2.2 Project background

In our work in the development of TEFM with spectroscopic detection techniques, we have two applications in mind: we want to investigate the distribution of fluorophores in tissue and measure the fluorescence properties of photosensitisers used for detection and treatment of malignant tumours. For both applications a photoactive substance is given to the patient and it accumulates in tumour tissue after some time (from some hours up to a day, depending on the substance). As the drug yields a characteristic fluorescence emission spectrum following violet light excitation, it can be used as a fluorescence marker of tumours (due to the tumour selective uptake of the photosensitiser).

Following excitation induced by red light the excess energy gained by these molecules can be transferred to oxygen, yielding cytotoxic singlet oxygen. Due to these properties these substances, called photosensitisers, are used in photodynamic therapy. The two agents examined in our studies are δ-amino levulinic acid (ALA)-induced Protoporphyrin IX (PpIX), and a haematoporphyrin derivative (Photofrin). ALA will be transformed to PpIX in the haem cycle in the cells. Both Photofrin and PpIX selectively accumulate in tumour tissue and catalyse photochemical reactions, making the tissue sensitive to light.

The mechanisms behind the selective accumulation in tumour tissue are not very well understood. To be able to better understand them it is of interest to study the spectroscopic properties of the fluorophores at a microscopic scale.

Another purpose is to study the distribution of different substances in newsprint. Due to an increased use of recycled paper along with higher demands set by new printing techniques it is of interest to examine newsprint at a microscopic level.

3. Materials and methods

The experimental arrangement used in our measurements is illustrated in Figure 1. The laser system used for excitation was an Ar-ion laser (Coherent Innova 400) pumping a Kerr-effect mode-locked Ti:Sapphire laser (Coherent MIRA 900). This system yielded 150 fs-light pulses at a wavelength of 792 nm with a repetition rate of 76 MHz. The average laser power used was 5 to 45 mW. Due to attenuation in the optics this was 1.5 to 13 mW at the sample. An average power of 10 mW corresponds to a peak power of 880 W. This gives a peak intensity in the focal area of $2.2 \cdot 10^{11}$ W cm^{-2} for a fully illuminated lens with NA=0.45 (the diameter at the focus is then 0.72 μm), regarding the laser beam idealised as a diffraction limited plane wave.

A conventional fluorescence microscope (Nikon Labophot II) equipped with a scanning stage was used. The laser light was led through the excitation light port and reflected by a dichroic mirror. The dichroic mirror reflected light at the laser wavelength, while it transmitted light in the wavelength region between 400 and 700 nm. Nikon CF Plan Apochromat microscope lenses (10x and 20x, with numerical apertures

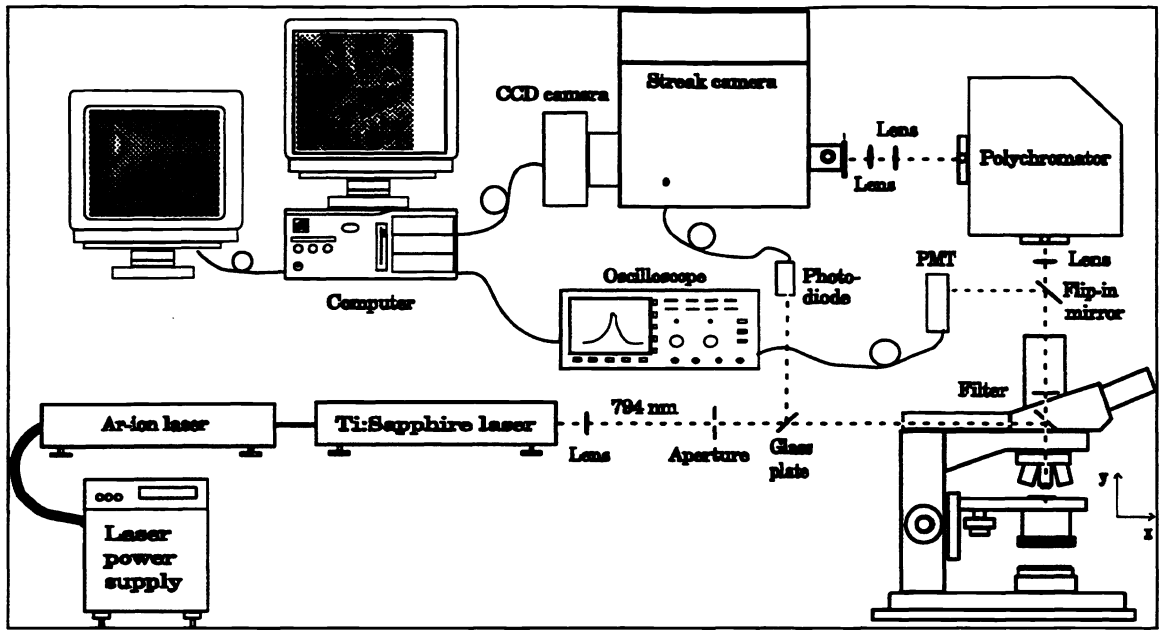

Fig. 1: Experimental set-up.

of 0.45 and 0.75, respectively) were used in the experiments. The fluorescence light was collected by the microscope lens and transmitted through the dichroic mirror before entering the detection system. The fluorescence signal was filtered with a BG39 Schott filter to suppress scattered laser light

For image scanning we used a scanning board (Märzhäuser Wetzlar EK32) controlled by a personal computer. A photomultiplier tube (Hamamatsu R928) was then used to detect the fluorescence light. The signal from the photomultiplier tube was fed to a digital oscilloscope (Tektronics TDS 520A). The oscilloscope was synchronised to the movements of the scanning stage. The real time curve obtained from the oscilloscope, corresponding to one scanned line, was read out via GPIB to the personal computer for further processing.

For measurements performed with the spectrometer, the light was filtered by a 2 mm BG39 Schott filter and focused onto the entrance slit of a polychromator (SPEX 270M), equipped with a 150 grooves / mm grating blazed at 500 nm. For detection a liquid nitrogen cooled CCD camera (EG&G Parc OMA 4000) was used. In some cases the exit plane of the polychromator was imaged using two standard camera lenses (50 and 135 mm focal length) onto the entrance slit of a streak camera tube (Hamamatsu, Model 1587-03), giving the spectral resolved image along the slit. A synchroscan streak unit (Hamamatsu M1955) was used to synchronise the streak scan with the laser pulses. A time- (and spectrally) resolved fluorescence spectrum could be recorded by a Peltier cooled CCD camera. A typical accumulation time of 100s was used for the spectrally resolved measurements.

We used another fluorosensor to record fluorescence spectra from macroscopic tissue samples using normal single-photon excitation. That system consists of a nitrogen-laser-pumped dye-laser, emitting light at 405 nm, an optical fibre guiding the excitation light to the sample as well as the fluorescence light back to a spectrometer connected to an air cooled CCD camera [3].

We investigated different types of samples:

- Rodent rat liver. The rat was injected with ALA (30mg/kg body weight) 3h before it was sacrificed and the organs removed. The removed organs were frozen in isopentane with carbonic ice and then

kept at a temperature of -18°C. No further staining or fixation procedures were adopted. Immediately before the measurements the samples were thawed and about 0.5 mm thick specimens were prepared with the use of a scalpel. The samples were placed on a microscopy glass and covered with a cover slip.

- Human serum containing Photofrin (Quadra Logic, Vancouver BC, Canada). Photofrin was dissolved in fresh human serum.
- Newsprint with different amounts of recycled fibres.

4. Results

In order to quantify the dispersion of the short laser pulses in the microscope optics, we measured the pulse width with an autocorrelator. Two microscope lenses (10x and 20x) were inserted in the beam. The pulse broadening effects were assumed to be the same for both microscope lenses. The autocorrelation curve [4] is shown in figure 2. The FWHM was 185 fs, yielding about 10% broadening in each of the lenses.

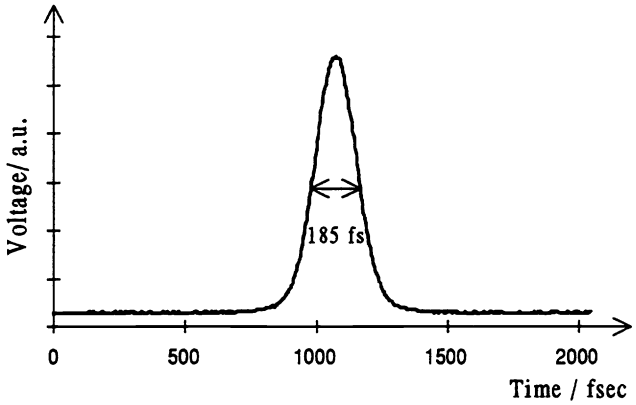

Fig. 2: Autocorrelation curve of the laser pulse passing two microscope lenses

A typical fluorescence spectrum of macroscopic rodent liver tissue, recorded with an optical multichannel analyser system, is shown in Figure 3. Thirty mg/kg body weight ALA had been administered

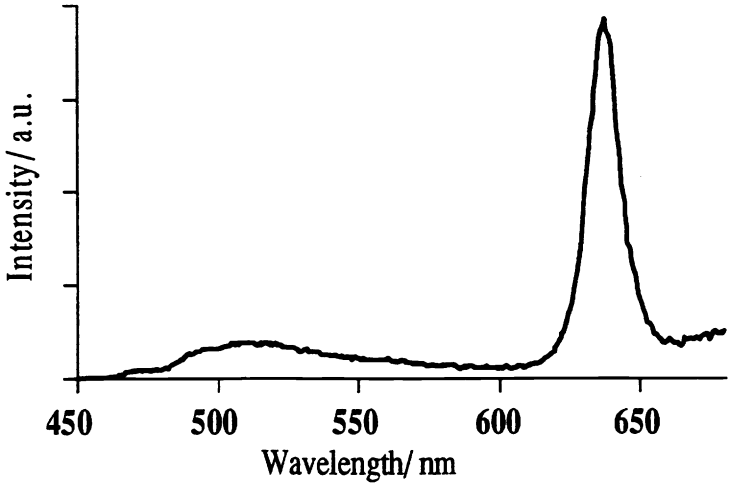

Fig. 3: Fluorescence spectrum of a rodent liver containing ALA, the excitation wave length was 405 nm.

intravenously 3 hours prior to the resection. This spectrum illustrates the type of fluorescence emission that can be measured from tissues. Two fluorescence regions can be distinguished: one broader range around 520 nm originating from endogenous fluorophores in tissue like elastin, collagen, NADH, vitamin derivatives etc. [5] (tissue autofluorescence) and one peak at 635 nm due to the ALA-induced protoporphyrin IX. Slices for microscopic studies were cut from the liver sample examined above. Images of the slices were recorded using a photomultiplier tube as a detector. An example is shown in Fig. 4a. A fluorescence spectrum was also obtained from a region of interest (Fig. 4b). The porphyrin peak has decreased in relation to the autofluorescence as compared with the macroscopically recorded spectrum.

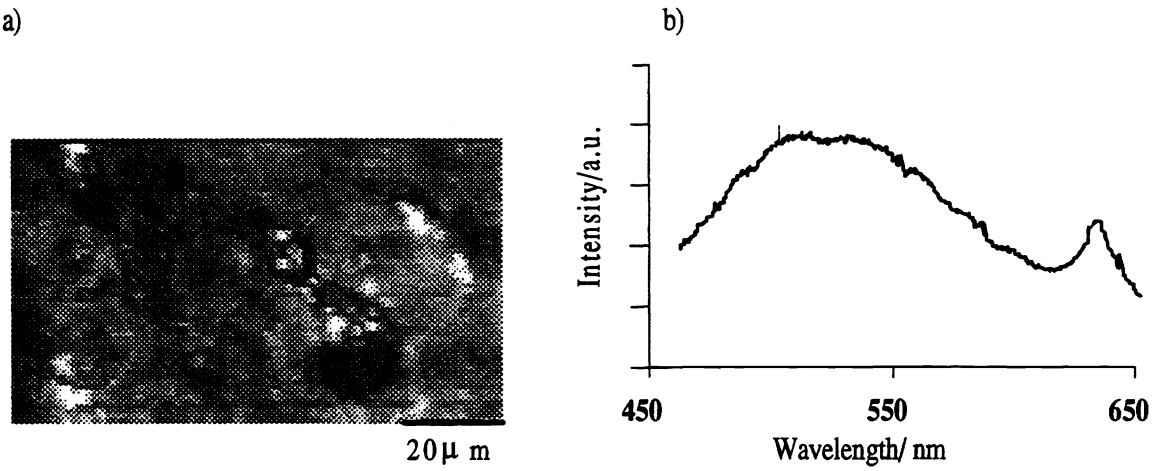

Fig. 4: a) Two-photon excited fluorescence image of a rodent liver sample containing ALA-induced protoporphyrin
b) Fluorescence emission spectrum of the same sample

The human blood serum containing Photofrin showed a typical spectrum (Fig. 5), as it is known for Photofrin. The serum itself is only weakly fluorescent upon two-photon excitation at 792 nm light, but the Photofrin peak at 630 nm is clearly visible.

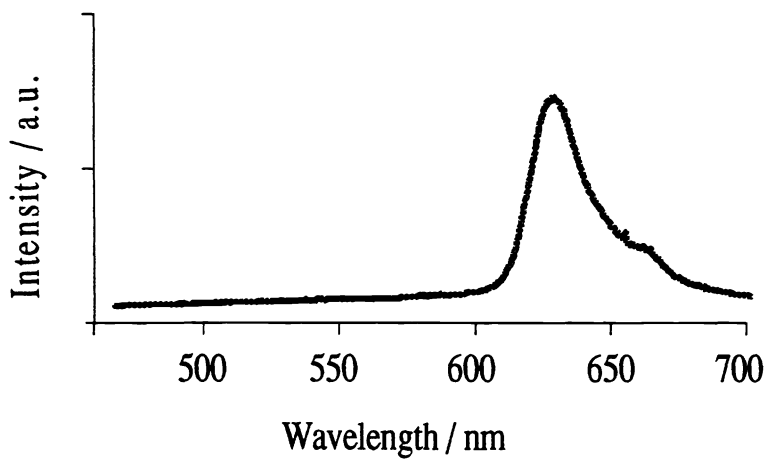

Fig. 5: Two-photon excited fluorescence spectrum of Photofrin dissolved in human blood serum.

The other type of samples studied was newsprint. A sample of newsprint was placed in the microscope and images were scanned. Fibres with a diameter of about 20 μm were clearly visible (see Fig. 6). Fluorescence emission spectra were recorded from two regions of interest, marked in the figures. Two spectra with completely different shapes resulted.

The time-resolved fluorescence decay curves of these two fluorophores were also measured and are shown in Fig. 7. These shapes have been identified in an earlier study [6] to be due to a fluorescing whitening agent and lignin, respectively. The strongly fluorescent whitening agents are not used in the production of newsprint, but are introduced into the paper with the recycled fibres.

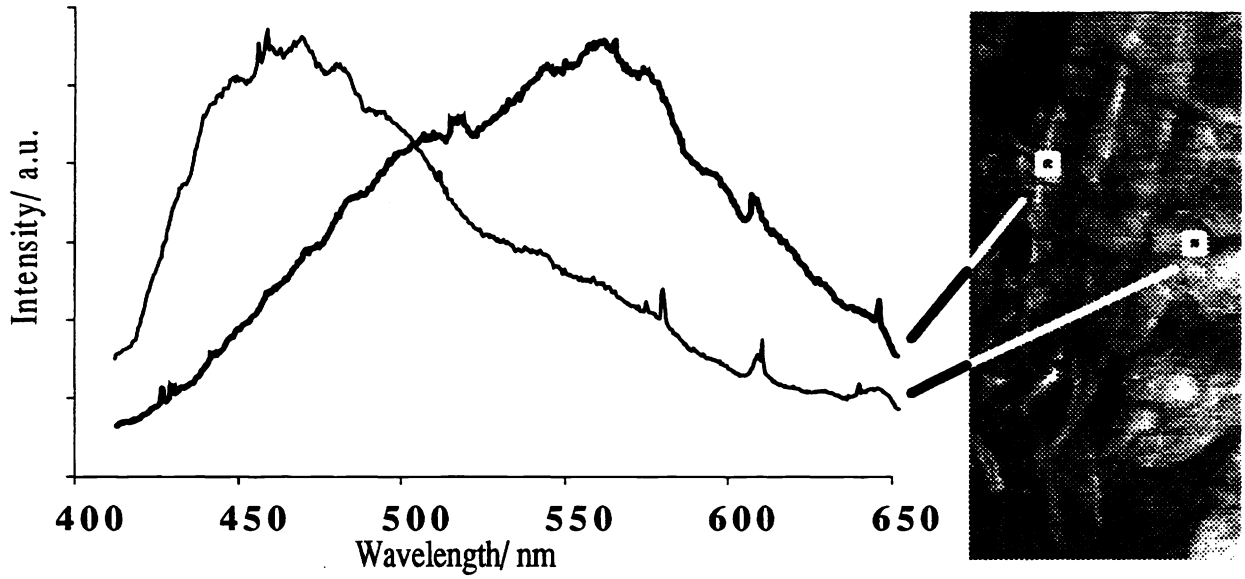

Fig. 6: Two-photon excited fluorescence spectra of newsprint fibres, recorded at the places as pointed out in the fluorescence image.

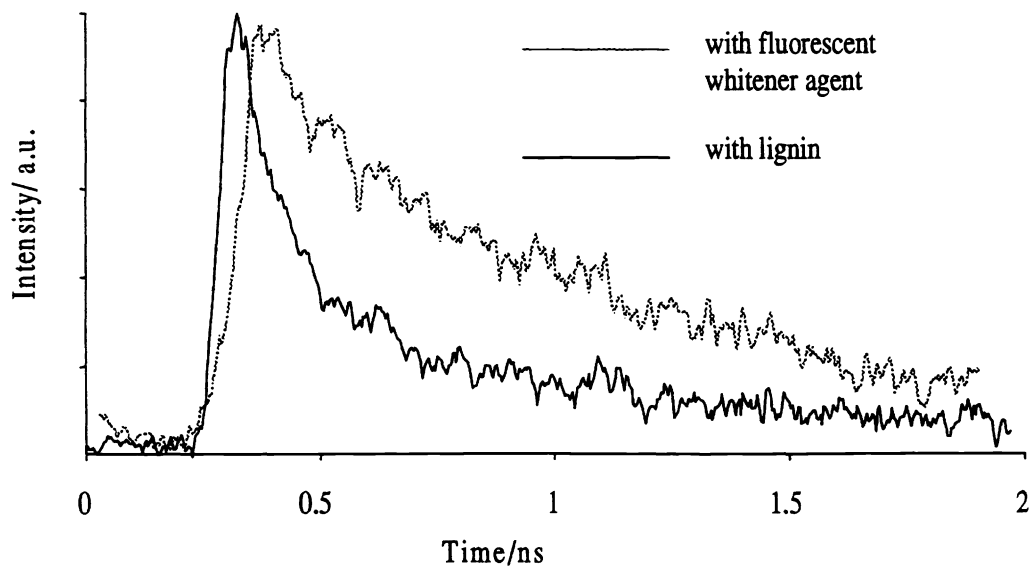

Fig. 7: Time-resolved fluorescence spectra of fibres in newsprint

5. Discussion and Conclusions

In the present study it has been possible to record fluorescence from tissue, photosensitiser and newsprint using two-photon excitation. It has been possible to measure emission spectra as well as scanned images.

Due to the low cross section for two-photon excitation the signals are weak and sensitive detection systems are required. Comparing the two spectra obtained from the liver sample (Fig. 3 and 4) a decreased porphyrin signal (in the case of two-photon excitation) is observed. The change in spectral shape can be explained in several ways. The concentration of protoporphyrin might be lower in the small region studied through the microscope than in the larger volume examined in the macroscopic fluorescence measurement.

Tissue spectroscopy will most probably also differ for one- and two-photon excitation. While the fluorescence emission characteristics should be independent of the excitation process for a single fluorophore, the spectral characteristics of tissue fluorescence should not be the same for the two excitation processes. The reason for this is that many fluorophores contribute, and the relative excitation probability for the various tissue fluorophores are likely to differ. In this case the two-photon excited cross section might be relatively lower for protoporphyrin than for the endogenous fluorophores contributing to the tissue autofluorescence (again compared with the one-photon excitation). In our measurements we could observe, that the signal was very sensitive to the penetration depth in the tissue.

It was possible to get an image of paper fibres using two-photon excited fluorescence microscopy similar to the image obtained with normal white light microscopy. The fluorescence intensity did not seem to be very sensitive to the topography of the sample surface. In the measurements it was also possible to distinguish lignin and fluorescent whitening agents due to their spectroscopic properties.

The main limitations with TEFM are the high peak power required and the comparable low signal levels provided. So far it has been possible for us to obtain signals from at the most a few μm depths. This might be due to the light scattering within the specimens reducing the peak intensity or just the low signal level.

TEFM might be developed to a useful tool to study the fluorophores contributing to tissue autofluorescence and the pharmacokinetics of the various photosensitisers under clinical trials for PDT. The distribution and accumulation kinetics of photosensitisers in the tissue are key properties to optimise the PDT outcome. For the drugs relevant for PDT, spectroscopic detection is required to be able to quantify the accumulation and to correctly map their distributions. Furthermore, the fluorescence properties of most of these drugs will slightly shift with the binding chemistry, resulting in an interesting method to improve the understanding of the origin of the selective accumulation of the photosensitisers in tumour tissue.

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