Two-photon fluorescence spectroscopy for identification of healthy and malignant biological tissues

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ABSTRACT

Two-photon fluorescence spectroscopy has been performed on rat skeletal muscles to investigate the effect of fixation processes on the micro-environments of the endogenous fluorophors in rat skeletal muscles. The two-photon fluorescence spectra measured for different fixation periods show a differential among those samples that were fixed in water, formalin and methanol, respectively. The results imply that two-photon fluorescence spectroscopy can be a potential technique for identification of healthy and malignant biological tissues.

Keywords: two-photon excitation, fluorescence spectroscopy, two-photon fluorescence, autofluorescence, tissue spectroscopy, fixation and fixatives.

1. INTRODUCTION

Viable cells have been well known to exhibit autofluorescence because of the intrinsic fluorophors bound to proteins within cells[1-8]. These endogenous fluorophores fluoresce in the visible spectral region. For example, the hydrated nicotinamide adenine dinucleotide (NADH & NADPH) and flavin molecules fluoresce in the blue and green spectral range when irradiated with UV light. Porphyrins in the human skin fluoresce in 590 – 640 nm. Usually, cellular autofluorescence has been considered a source of unwanted interference for many applications as they may obscure the fluorescence coming from the probe of interest. However, these native fluorophors can be put into good use to produce superb images of cellular structures without the need for adding a fluorescent label. For example, NADP & NADPH is concentrated in the mitochondria and so can be used as a maker of mitochondria in cells. Furthermore, these native fluorophors display well-defined spectral features that characterise the local environment and the state of cells, and therefore can be used as a maker to distinguish between healthy and abnormal tissue *in vivo*. Their spectroscopy is sensitive to the micro-environment of pH, redox potential, bonding sites, polarity, ion concentration, etc. Many researches have applied autofluorescence spectroscopy for diagnosis in biological tissues and some preliminary results have been achieved by using single-photon excitation[2,3,6,7]. It has been shown that flavins, NADP & NADPH and porphyrins exhibit spectral changes when transforming from the oxidised to reduced state.

Biological tissue is also known to be highly diffusive, which makes it difficult to obtain the information required at a significant depth into tissue and to perform useful microscopic and spectroscopic analyses. One of the methods for solving this problem is to use two-photon excitation produced by an infrared ultrashort pulsed laser beam. The use of infrared wavelength excitation significantly reduces multiple scattering and leads to a deeper penetration. In addition, because of the quadratic dependence of the excitation intensity, two-photon excitation results in an intrinsic optical sectioning effect that offers better spatial and axial resolution, less photo bleaching and less photo-damage. Because the two-photon method

offers a variety of advantages in fluorescence microscopy over single-photon excitation, the application of two-photon excitation for medical diagnosis has been proposed, such as two-photon biopsy[9], two-photon tissue microscopy and spectroscopy[10,11].

As a preliminary step towards *in vivo* non-invasive diagnosis, characterisation of the difference between normal and abnormal tissues *in vitro* is a necessity. Biological specimen are normally fixed or preserved in various selective fixatives for many *in vitro* research applications. The fixation process on tissues may incur any changes in autofluorescence, including the lifetime, spectrum or intensity as they may change or damage the structure or micro-environment of cells and tissues. This research involves two-photon spectroscopic measurements on rat skeletal biological tissues that were prepared using various fixatives, and demonstrates a differential among these biological tissues.

2. TWO-PHOTON TECHNIQUE

2.1 Single-photon Absorption

Fluorescence microscopic imaging and spectroscopy appear to be a valuable method for analysing small biological samples. The common and frequently used method for excitation of fluorescence is the single-photon absorption, in which a fluorophor absorbs a higher energy photon and fluoresces a lower energy photon (Fig 1-1). The fluorescence spectrum exhibits a shift to a longer wavelength range, named, the Stoke shift. Single-photon absorption has played an important role in fluorescence microscopic imaging and spectroscopy and has been demonstrated to be an effective method for the excitation of fluorescence. Most confocal microscopes are optimised for single-photon excitation. However, because single-photon excitation operates at short wavelengths (UV or visible), when applied to highly diffusive biological tissues, it is difficult to obtain the useful information at a significant depth into tissue and to perform useful microscopic and spectroscopic analyses. Its application is then limited by its ability of penetration.
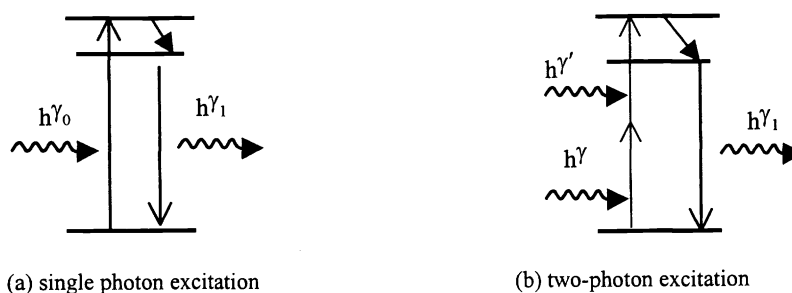

(a) single photon excitation   (b) two-photon excitation

Fig 1. Single-photon and two-photon excitation.

2.2. Two-photon Absorption and Two-photon Fluorescence

Two-photon excited fluorescence is produced by simultaneous absorption of two photons that have lower photon energy (Fig 1-1). Since the energy of a photon is inversely proportional to its wavelength, the two photons should be about twice the wavelength required for single-photon excitation. For example, a fluorophore that normally absorbs ultraviolet light (~350 nm) can also be excited by two red photons (~700 nm) if they reach the fluorophore at the same time (within 10^{-15} seconds). The use of near infrared wavelength excitation significantly reduces multiple scattering and leads to a deeper penetration. It also offers a full optical window from the UV to visible range for collection of emitted fluorescence due to the availability of an infrared tunable laser.

Because two-photon excitation depends on simultaneous absorption of two incident photons, the resulting fluorescence emission depends on the square of the excitation intensity. The theory of two-photon absorption was proposed as early as in 1931 by Goppert-Mayer. The time-averaged two-photon molecular absorption rate can be expressed as[10,12-15]:

$$\phi_{2p} = 0.56 \frac{1}{\tau f} \frac{P_{ave}^2}{(\eta \omega)^2 F^2} \sigma_{2p} \tag{1}$$

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where 0.56 holds for hyperbolic-secant shaped pulses, P_{ave} is the time-averaged power, τ represents the pulse duration, f is the repetition rate of the pulses, $\hbar\omega$ is incident photon energy, F the focal area of the lens and σ_{2p} the two-photon cross-section.

Because of the requirement of the simultaneous absorption of two photons, the probability for generating two-photon fluorescence is small. The two-photon absorption cross-section is more than 10 orders of magnitude smaller than single-photon absorption cross-section. To obtain a significant number of two-photon absorption events, the photon density must be approximately a million times what is required to generate the same number of single-photon absorption. This means that extremely high laser powers are required to generate a lot of two-photon excited fluorescence. Instead of using a high power CW laser, a mode-locked pulsed laser is normally used. The peak power of the pulse is high enough to generate significant two-photon excitation while the average power is fairly low. This is particularly advantageous in biological applications since low average power causes less photodamage and photobleaching - two of the most important limitations in fluorescence microscopy of living cells and tissues. The pulse duration is typically from ~ 100 femtosecond to ~ 1 picosecond. The peak power can be up to 10 MW while the average power is limited to less than 10 mW, which is just slightly greater than that used in confocal microscopy.

The quadratic dependence of the two-photon excitation intensity results in the fluorescence excitation only at the focal point. This localisation of two-photon excitation provides an inherent optical sectioning property for three-dimensional imaging and gives rise to many of the significant advantages associated with two-photon excitation microscopic imaging and spectroscopy over confocal microscopy. In a confocal microscope, fluorescence is excited through the sample, but only the signal from the focal plane passes through the confocal pinhole, so background-free data can be collected. By contrast, two-photo excitation generates fluorescence only at the focal point, so there is no background and no pinhole is required. Because there is no out-of-focus absorption, more of the excitation light penetrates through the sample to the focal point. This leads to greatly increased sample penetration-generally at least two to three times deeper than is possible with confocal microscopy. Photodamage and photobleaching is also minimised and confined to the focal plane. The optical sectioning property of two-photon excitation allows it to be used as a choice for medical biopsy.

3. EXPERIMENTS

Fig 2 is the schematic diagram of the two-photon fluorescence microscope used in this work. This system includes a femtosecond pulsed laser (Tsunami pumped by Millenmia, Spectra-Physics) and a confocal laser scanning microscope (Fluoview, Olympus). The femtosecond pulsed laser has a wavelength tuning range from 690 nm to 1060 nm, which provides a source for two-photon microscopy. The scanning unit within the confocal microscope provides a mechanical mechanism for three-dimensional imaging and spectrum measurements. The laser beam is coupled first to the scanning unit through the optical coupling box, and then to the microscope through a collimated lens, and is finally focused on to the sample by a high numerical aperture water-immersion objective (Olympus, NA=1.25, 60X). The generated fluorescence within the sample is collected by the same objective and travells back to the scanning unit for detection by a PMT detector installed in the scanning unit. A series of narrow bandpass interference filters (10 nm) were used to measure the fluorescence spectra. These filters have a FWHM bandwidth of ~ 10 nm and are inserted in the fluorescence emission path between the objective and the detector. Measurements were taken at a 10 nm wavelength separation. The excitation wavelength used for spectrum measurements was 800 nm.
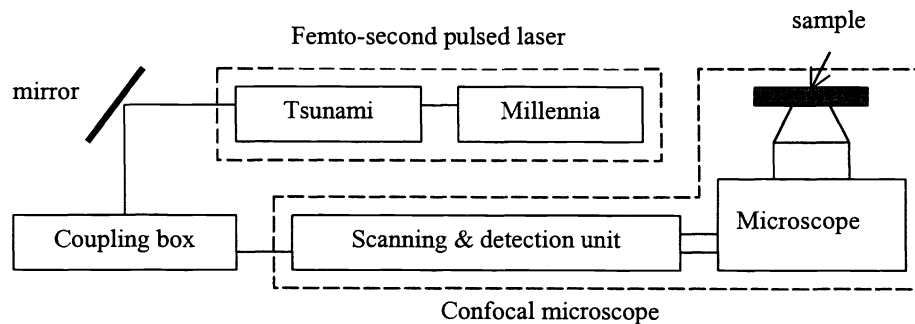

Fig. 2 Schematic diagram of the two-photon fluorescence microscope

The rat muscles were cut from the rats, and soaked in water, formalin and methanol, respectively. Spectra measurements were performed for fixation periods of 1, 2, 3 and 4 days, respectively. Fresh muscles without any fixing processes were also prepared for comparison.

4. RESULTS AND DISCUSSION

Fig. 3 shows a two-dimensional fluorescence image of the fresh rat skeletal tissue and its two-photon fluorescence spectra. The image size is 50x50 μm. It includes three well resolved tissue fibres that show strong but not uniform fluorescence. The spectra clearly show two peaks of emission at around 470 nm and 540 nm. These two peaks are attributed to two types of fluorophors existed in the tissues, NADH (470nm) and flavin (540nm).
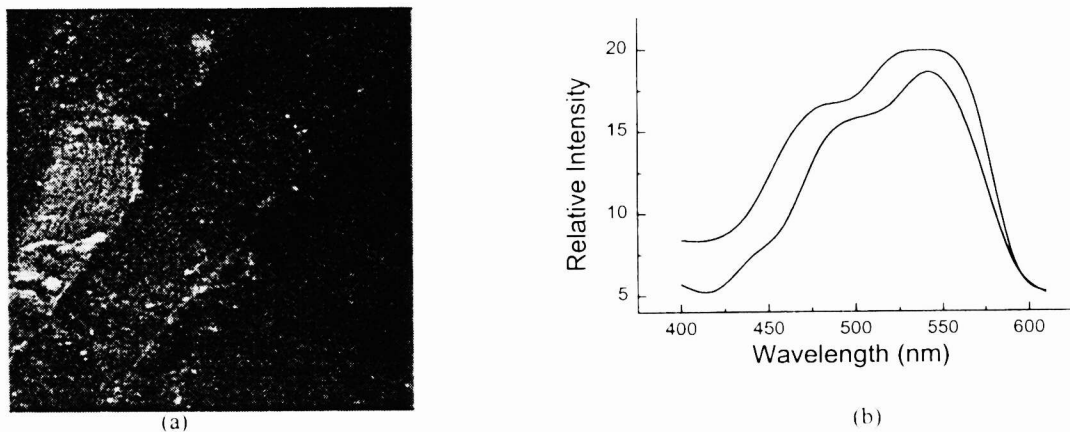

(a) (b)

Fig. 3 Two-photon fluorescence a) image and b) spectrum of fresh rat skeletal tissue

Fig 4 shows the dependence of the fluorescence intensity on the excitation power at one autofluorescence peak 540 nm. The slope of the log-log plot line is $1.90^{\pm}0.1$, which indicates a quadratic dependence of the fluorescence signal on the excitation power and confirms the fluorescence excitation by two-photon absorption (see equation (1)).
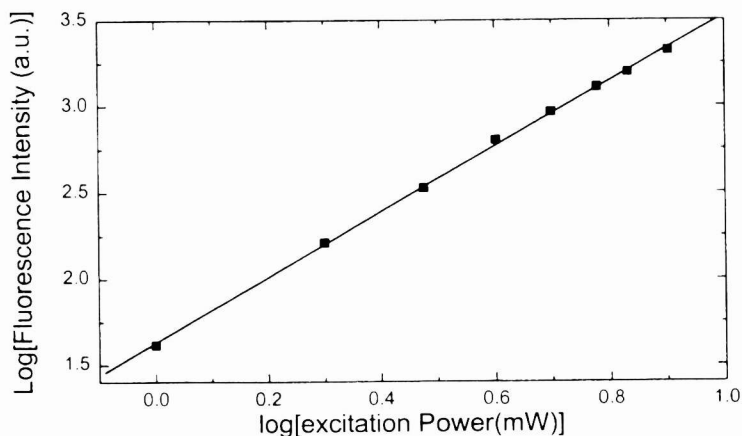

Fig 4 Fluorescence intensity vs the excitation power.

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Fig 5 gives the results of the measured spectra for rat skeletal tissues fixed in fixatives of water, formalin and methanol for fixation periods of 1, 2, 3 and 4 days. These results show that when samples were fixed in formalin and water fixatives, their fluorescence spectra show little change, as two fluorescence peaks are persistent on the spectra. However, it is not the case for those fixed in methanol. The spectra obtained from the methanol-fixed sample show only one peak at around 540 nm, the peak that was observed at 470 nm disappeared in methanol-fixed samples, indicating that the use of methanol as a fixative can significantly change the fluorescence spectrum of tissue compared to formalin. The spectra from formalin-fixed tissues show similar characteristic to those obtained from fresh rat muscle, indicating that formalin is the least invasive fixative for preservation of tissue. Water is a natural biological environment within tissues, and can preserve tissues well for a short period, as indicated in Fig 5. However, other results showed that the spectra of rat tissues change significantly when fixed in water for longer than 7 days and therefore is not a good fixative.
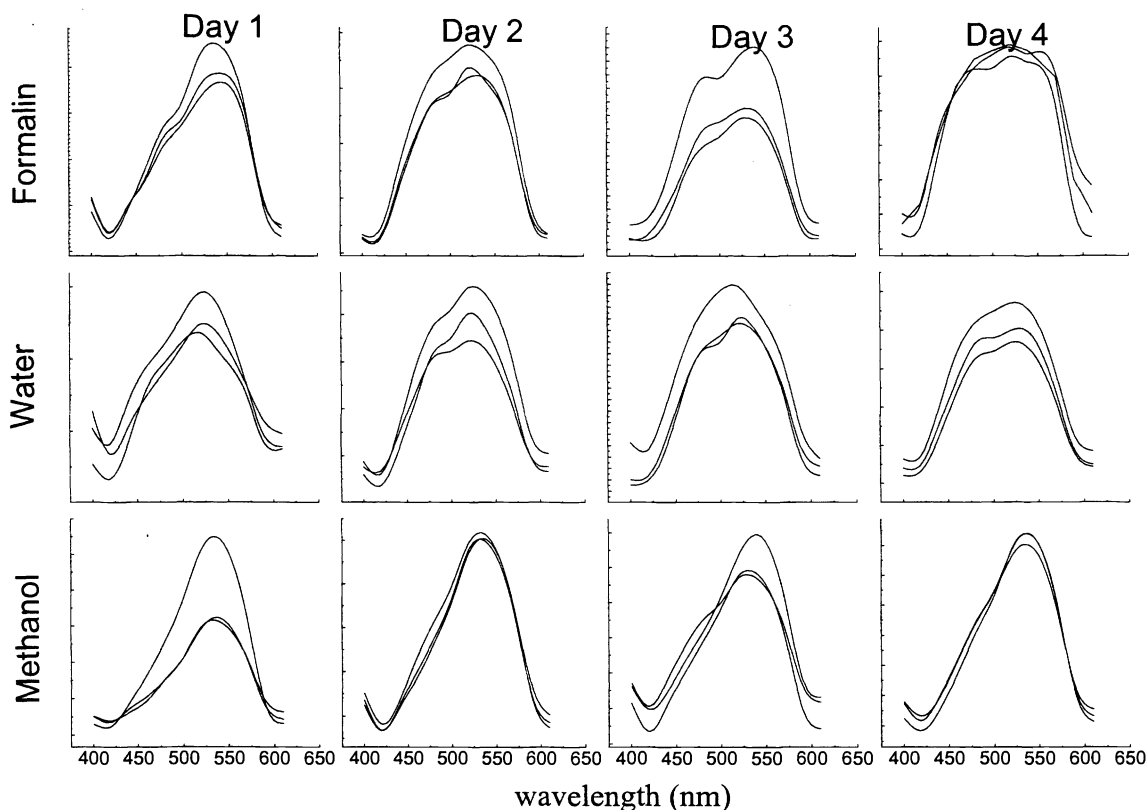

Fig 5 The measured two-photon fluorescence spectra for rat skeletal tissues fixed in water, formalin and methanol, respectively, at fixation periods of 1, 2, 3 and 4 days

5. CONCLUSSION

We have shown the difference of two-photon fluorescence spectroscopy on rat skeletal muscles that were fixed in water, formalin and methanol, respectively. It is shown that the two-photon fluorescence spectra do not change much for those tissues fixed in formalin and water, but change significantly for those fixed in methanol. The spectra from formalin- and water-fixed tissues are similar to those from fresh muscles for a fixation period up to 4 days. In order to develop two-photon fluorescence spectroscopy for *in vivo* non-invasive diagnosis, formalin is a better fixative for preserving samples in *in vitro* researches than methanol since formalin does little effect on spectroscopy of its fixed sample.

ACKNOWLEDGMENTS

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