Effect of handling and fixation processes on fluorescence spectroscopy of mouse skeletal muscles under two-photon excitation

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We investigated the effects of handling and fixation processes on the two-photon fluorescence spectroscopy of endogenous fluorophors in mouse skeletal muscle. The skeletal muscle was handled in one of two ways: either sectioned without storage or sectioned following storage in a freezer. The two-photon fluorescence spectra measured for different storage or fixation periods show a differential among those samples that were stored in water or were fixed either in formalin or methanol. The spectroscopic results indicate that formalin was the least disruptive fixative, having only a weak effect on the two-photon fluorescence spectroscopy of muscle tissue, whereas methanol had a significant influence on one of the autofluorescence peaks. The two handling processes yielded similar spectral information, indicating no different effects between them. © 2000 Optical Society of America

OCIS codes: 170.6510, 170.6280, 170.6930, 170.4580, 170.2520, 170.7160.

1. Introduction

Fluorescence microscopic imaging and spectroscopy are valuable methods for analyzing small biological samples and for studying the electronic structures of molecular excited states.[1,2] Conventional fluorescence excitation is based on single-photon absorption in which a fluorophor absorbs a higher-energy photon and fluoresces a lower-energy photon. This technique has been demonstrated to be effective and has played an important role in fluorescence microscopic imaging and spectroscopy.[1,2] However, because single-photon excitation usually operates at UV or visible wavelengths, it may cause UV damage and results in limited penetration when applied to diffusive biological tissues. Therefore it is difficult to perform useful microscopic and spectroscopic analyses at a significant depth into tissue.

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Received 29 March 2000; revised manuscript received 7 July 2000.

0003-6935/00/346312-06$15.00/0

The above problems can be solved by use of two-photon excitation in which a visible fluorescence photon is produced by the simultaneous absorbance of two incident infrared photons.[3–7] Furthermore, because of the quadratic dependence on the excitation intensity, two-photon excitation results in an inherent optical-sectioning property that offers axial resolution for three-dimensional imaging[4] and better background-fluorescence rejection.[6,7] Because of these advantages, two-photon excitation techniques that use autofluorescence generated from the endogenous fluorophors within cells[8–14] have been proposed for use in noninvasive clinical diagnosis, such as two-photon biopsy[15] and two-photon tissue microscopy and spectroscopy.[16–18]

Cellular autofluorescence in mammalian cells is found to be dominated by the two distinct endogenous fluorophors: the pyridine nucleotide [NAD(P)H] and flavin compounds bound in mitochondria and cytoplasm.[19–21] These two fluorophors have emission spectra between the wavelength ranges of 400–500 and 500–600 nm and require separate excitations at wavelength ranges between 360–365 and 440–450 nm under single-photon excitation.[19–21] However, in our previous report[4] it was found that these fluorophors could be excited simultaneously by two-photon absorption at a wavelength of 800 nm because of the different transition-selection rules involved in both cases. This unique characteristic offered by two-photon excitation provides the possibility of us-

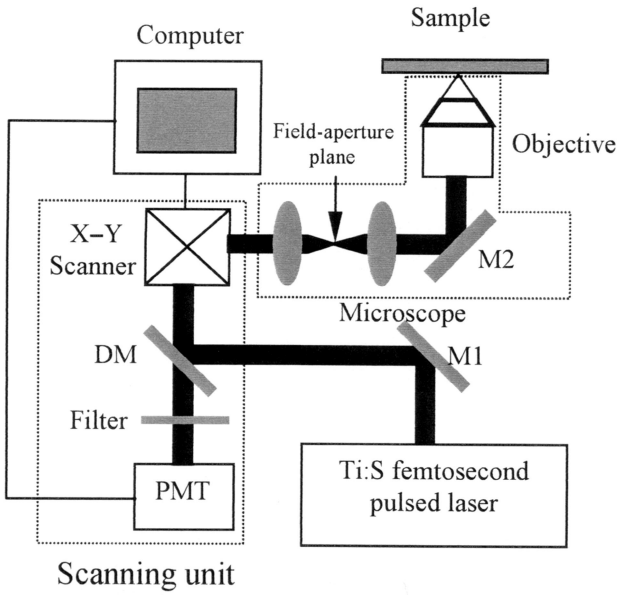

Fig. 1. Schematic diagram of the two-photon fluorescence microscope. DM, dichroic mirror; M1 and M2, mirrors; PMT, photomultiplier tube.

ing the two endogenous fluorescence peaks as a marker to distinguish between healthy and abnormal tissues *in vivo*. As a first step toward *in vivo* noninvasive diagnosis by use of two-photon technology, we performed two-photon fluorescence spectroscopy measurements on mouse skeletal muscle to investigate the changes of the two autofluorescence peaks with respect to different handling and fixation processes that are routinely used in pathological research and clinical diagnosis. The results showed that autofluorescence was altered in a time- and a fixative-dependent manner.

This paper is arranged as follows: In Section 2, a description of the details of the experimental setup and the sample-handling and sample-preparation processes is given. The measured results of two-photon fluorescence and its spectroscopy on mouse skeletal muscle, together with a discussion of the results, are given in Section 3. Several conclusions about the selection of the fixatives and the handling techniques are summarized in Section 4.

2. Experiments

A schematic diagram of the two-photon fluorescence microscope is shown in Fig. 1. This system includes a femtosecond pulsed laser (Spectra-Physics) and a confocal laser-scanning microscope (Olympus). The femtosecond pulsed laser had an output pulse width between 70 and 100 fs and a wavelength-tuning range from 690 to 1060 nm, which provided a source for two-photon microscopy and spectroscopy. The scanning unit within the confocal microscope, which included an x–y scanning mirror and a photomultiplier tube, provided a mechanical mechanism for three-dimensional imaging and spectrum measurements. The laser beam was coupled first to the x–y scanner through mirror M1 and a dichroic mirror, then to the microscope through a pair of collimated lenses, and finally was focused onto the sample by a high-numerical-aperture (high-NA) water-immersion objective (Olympus, NA = 1.25, 60×). The fluorescence generated within the sample was collected by the same objective and returned to the scanning unit for detection by a photomultiplier tube. The original dichroic mirror in the scanning unit was replaced by a short-pass dichroic mirror (which transmits UV to visible light and reflects the near infared at 45°) for optimum two-photon operation. Because of the dispersion of the optics in the incident path, the actual pulse width delivered to the sample was approximately 300 fs. A series of narrow-bandpass interference filters were used to measure the fluorescence spectra. These filters had a FWHM bandwidth of approximately 10 nm and were inserted in the fluorescence-emission path between the objective and the detector. Measurements were taken at a 10-nm wavelength separation. For all the measurements presented in this study, the fluorescence-excitation wavelength used was 800 nm for simultaneous excitation of the two fluorophors, and the excitation power delivered to the sample was limited to approximately 6 mW to avoid photodamage.

Skeletal muscle was taken from Balb/e mice that were euthanized by cervical dislocation. The material used in these experiments was surplus to other experiments being performed in the laboratory. All experiments were conducted in accord with National Health and Medical Research Council animal ethics guidelines. The skeletal muscles were dissected free of surrounding tissues and prepared in two groups by use of two different handling processes. The first group (group 1) was prepared by means of block cutting without prestorage, which means that the muscle tissue was cut into bulky forms and then placed directly into water, formalin, or methanol for a certain period of time without prestoring it in a freezer. The second group (group 2) was prepared by means of slice sectioning with prestorage, which means that the muscle tissue was stored in a freezer by use of O.C.T. (optimal cutting temperature) Compound (Tissue-Tek, Miles Inc, Indianna, USA) for a period of time, then sliced into 50-μm-thick sections at −20 °C in a cryostat, and finally placed in water, formalin, or methanol. Spectral measurements were performed following storage–fixation periods of 1 to as many as 6 days. Spectral measurements from fresh muscle that had not undergone any fixation were also conducted for comparison.

3. Results and Discussion

A. Fresh Tissues

Figure 2 shows a two-dimensional fluorescence image of fresh mouse skeletal muscle and its two-photon fluorescence spectra that was excited at a wavelength of 800 nm. The image size is 200 μm × 200 μm. It includes several well-resolved muscle fibers that show strong but not uniform fluorescence. The nonuniformity of the fluorescence emission indicates the

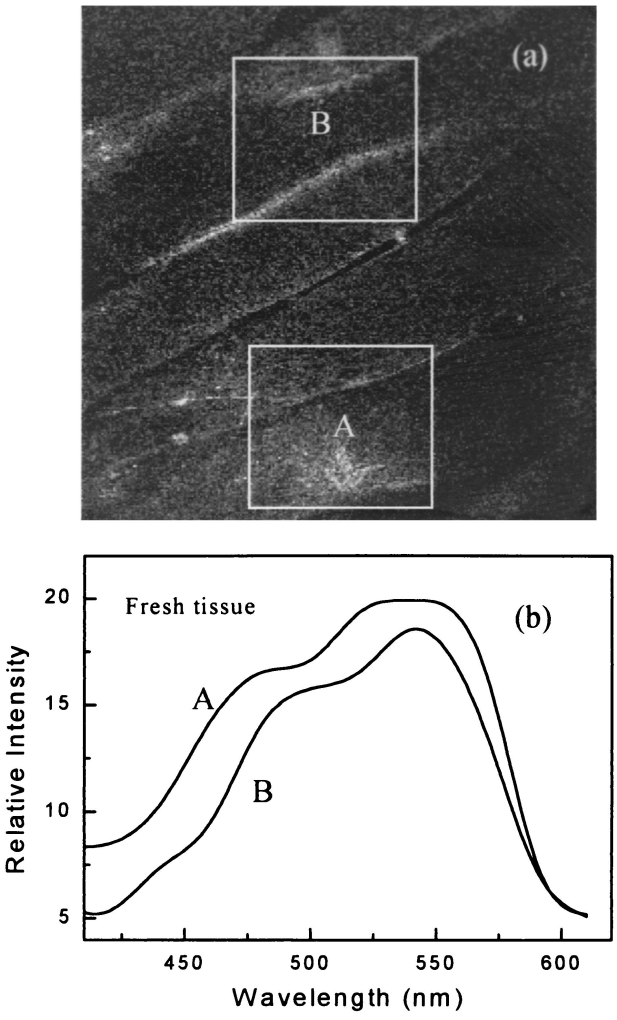

Fig. 2. Two-photon fluorescence (a) image and (b) spectrum of fresh mouse skeletal muscle. The squares marked A and B in (a) are the measured spots that correspond to the two similarly labeled spectra in (b).

localization of the endogenous fluorophors within the muscle fibers. The two spectral curves shown in Fig. 2(b) were measured at two separate spots of the tissue; both clearly show similar spectral characteristics, with two emission peaks at approximately 470 and 540 nm. These two peaks are attributed to two types of fluorophors that exist in the tissues: NAD(P)H (470 nm) and flavin (540 nm). The slight difference in magnitude between these two spectral curves results from the variation of fluorophor concentrations at these measured areas. Note that the positions of the two fluorescence peaks found in this study are slightly different from those shown in our previous report[4] in which the two fluorescence peaks were situated at 450 nm [NAD(P)H] and 550 nm (flavin). This difference is probably caused by the different sample-preparation processes.

B. Effects of the Handling and the Fixation Processes

Because moisture can affect fluorescence in tissue significantly, the measurements of fluorescence were performed immediately after the sample was taken out of the fixative to ensure that the sample was still moist with the fixative, and all measurements were carried out under the same conditions. Figure 3 shows the measured fluorescence intensity as a function of the storage–fixation time for water and the two fixatives for group 1 samples and fresh muscle at the excitation wavelength of 800 nm. It is noteworthy that the fluorescence intensity is sensitive to the measurement conditions.

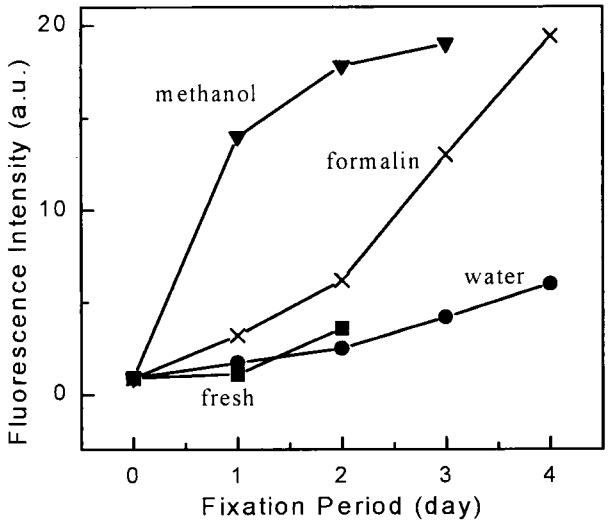

Fig. 3. Measured two-photon fluorescence intensity plotted as a function of the storage or the fixation period for mouse skeletal tissues that were stored in water or were fixed in formalin or methanol.

The results presented in Fig. 3 are the relative average fluorescence intensity of an area of 50 μm $\times$ 50 μm under the same measurement conditions that were described in Section 2. The initial point at day zero is the intensity for fresh muscle tissue. It is shown that the fluorescence intensity increases with the storage–fixation period for water and the two fixatives; in particular, methanol fixation results in a very rapid increase. The fluorescence intensity increases by 1 order of magnitude over the fixation period of 2 days for the formalin and the methanol fixatives, whereas the effect of water is less pronounced. This phenomenon is not completely understood; it is probably due to the chemical or the physical processes involved in fixation that destroy other structures, such as enzymes. It is also shown in Fig. 3 that the fresh sample exhibits an increase in fluorescence with time; this is probably because water has a quenching effect on fluorescence. As the sample was drying out, its fluorescence increased.

To verify that the fluorescence emission under 800-nm excitation was caused by two-photon absorption, we measured the dependence of the fluorescence intensity on the excitation intensity at a fluorescence wavelength of 540 nm under excitation at the wavelength of 800 nm. The results measured for group 1 (fixed in formalin for 2 days) are shown in Fig. 4. The slope of the log–log plot is 1.90 $\pm$ 0.1, which

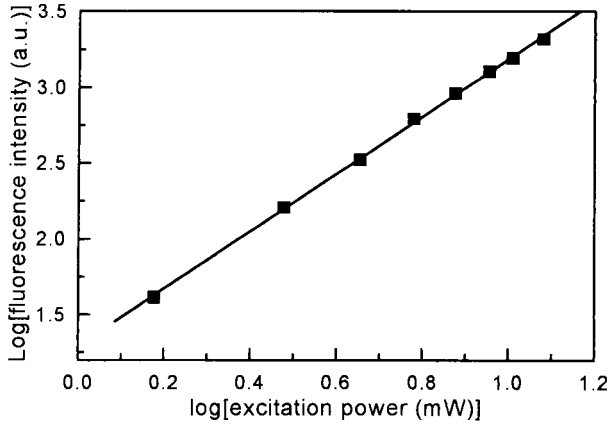

Fig. 4. Fluorescence intensity plotted as a function of the excitation power measured at the fluorescence peak at the wavelength of 470 nm.

indicates a quadratic dependence of the fluorescence signal on the excitation power and confirms the fluorescence excitation by two-photon absorption. Similar results were obtained for group 2 samples under the same experimental conditions.

The measured results of the two-photon fluorescence spectra for group 1 samples, which were stored in water or fixed in either formalin or methanol for 1, 2, 3, or 4 days, are shown in Fig. 5. The three spectra included in each set of curves were measured at three different spots within an area of 200 μm × 200 μm and show similar spectral characteristics. The slight differences in magnitude among the three curves in each set was due to slight differences in the fluorophor concentrations at those selected spots. Figure 5 shows that, when samples were stored in water or fixed in formalin, their fluorescence spectra exhibit little change with the periods because two fluorescence peaks are persistent in the spectra. These spectra also show spectroscopic characteristics similar to those obtained from fresh muscle tissue [see Fig. 2(b)]. However, the results for those muscle tissues fixed in methanol are different. The spectra obtained from the methanol-fixed samples show only one distinct fluorescence peak at a wavelength of 540 nm; the peak at the wavelength of 470 nm that was observed in fresh muscle becomes much less distinct and eventually disappears.

Figure 6 displays the measured two-photon fluorescence spectra for group 2 samples, which were stored in water or fixed in either formalin or methanol for 1, 2, 3, or 4 days and were analyzed under the same measurement conditions as those shown for Fig. 5. Spectral characteristics similar to those seen from Fig. 5 are observed.

C. Discussion

The reason that the two-photon fluorescence spectra vary differently for water and the two fixatives is due

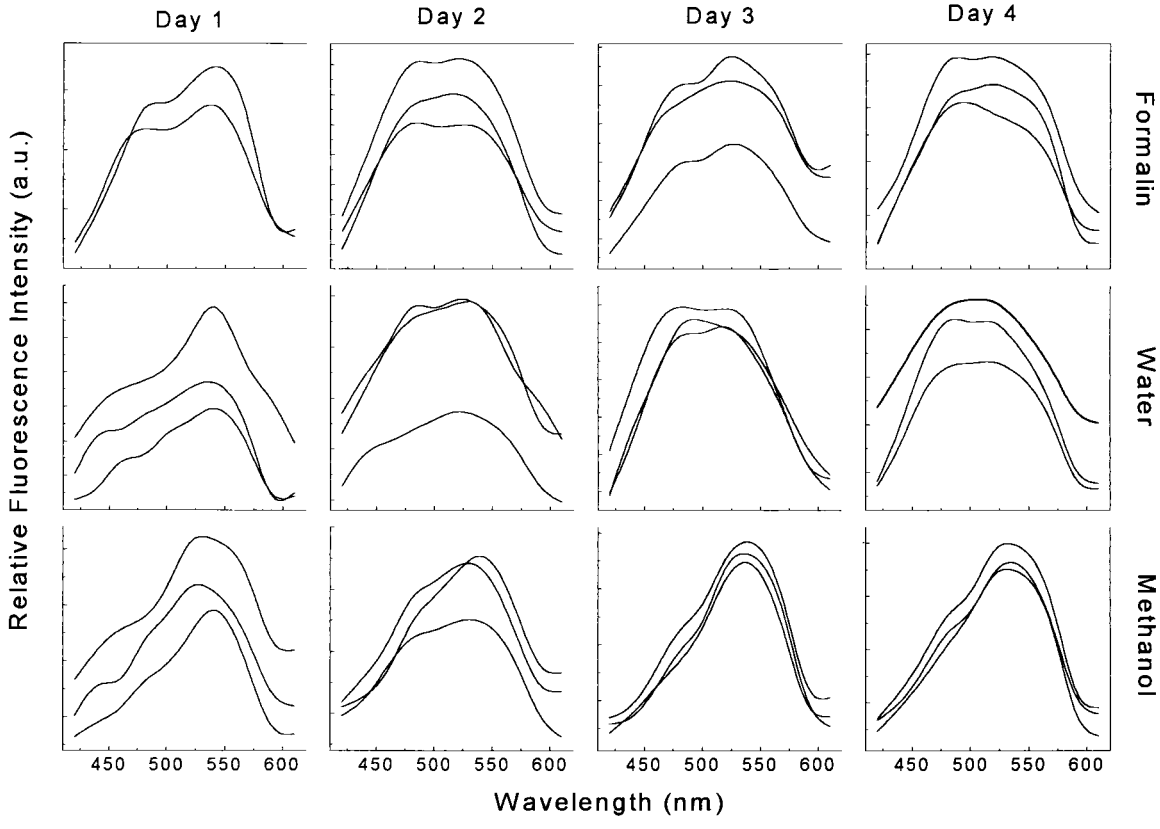

Fig. 5. Measured two-photon fluorescence spectra for group 1 samples that were stored in water or were fixed in formalin or methanol for 1, 2, 3, or 4 days.

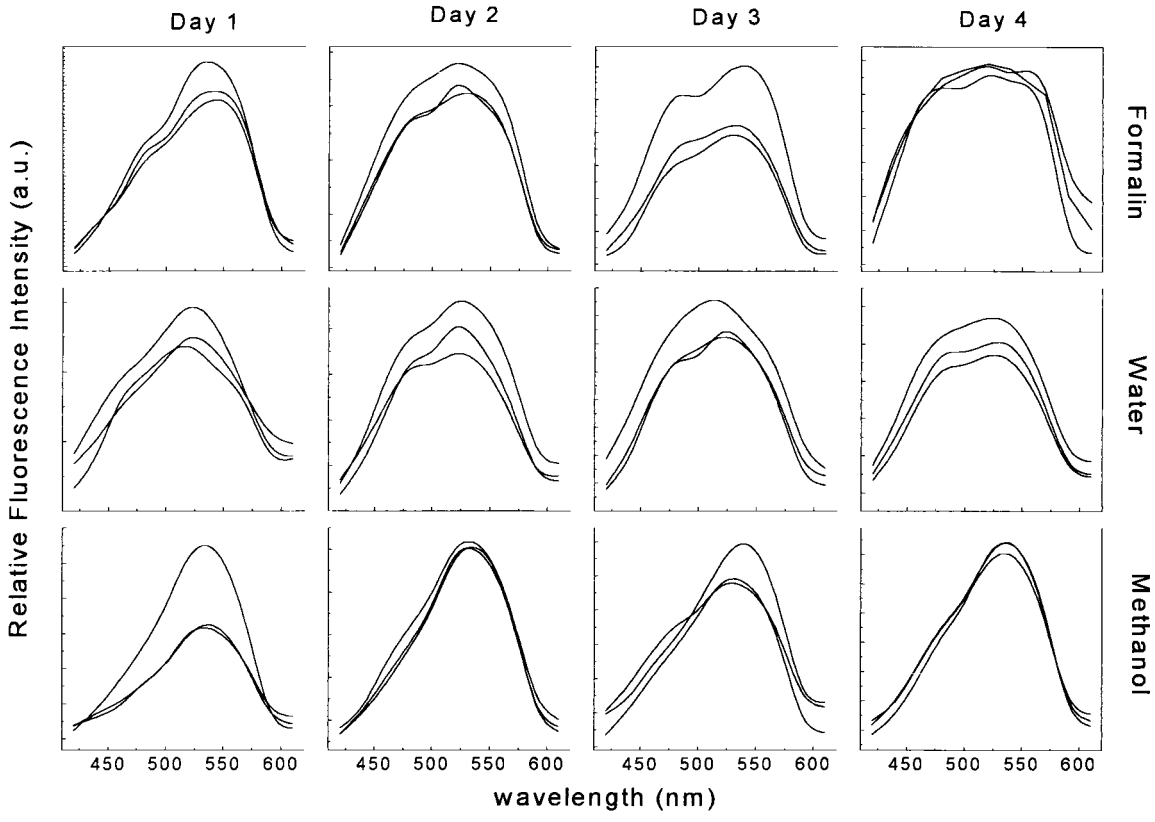

Fig. 6. Measured two-photon fluorescence spectra for group 2 samples that were stored in water or were fixed in formalin or methanol for 1, 2, 3, or 4 days.

to the different underlying fixation mechanisms.[22] Formalin is a cross-linking fixative that chemically forms covalent cross-links with proteins. It has good and rapid penetration into tissue and provides good tissue and protein preservation. Tissues can therefore be preserved in formalin for a relatively long time (as long as 2 weeks) without incurring significant changes in the microenvironments of the native fluorophors and their autofluorescence spectra.

Methanol is a coagulating fixative that rapidly changes the hydration state of the cellular component. It has good tissue penetration but is relatively less robust in preservation compared with formalin. In addition, methanol may extract water and proteins from the cell membranes in the tissue and damage the microenvironments, thus affecting the states of the endogenous fluorophors and their fluorescence spectra. In the methanol-fixation process the autofluorescence peak at the wavelength of 470 nm varies in a more pronounced way than does the peak at the wavelength of 540 nm. This is because NAD(P)H is a hydrated protein and the states of its microenvironments are more reactive to methanol than are those of flavins.

Water is not usually considered to be a fixative because it does not fix tissue. It constitutes a natural biological environment within tissue and thus does not degrade tissue for a short period (as long as 4 days), as indicated in Fig. 5. However, it was found (see Fig. 7) that the spectra of mouse tissues changed significantly when left in water for longer than 4 days, most likely because of degradation of the tissue in the aqueous, nonfixing conditions.

The results obtained from the two groups of samples prepared by use of the two different handling processes indicate no significant difference in two-photon spectroscopy between these two handling processes. However, slice sectioning with the O.C.T. compound prestorage method allows the sample to be kept in a refrigerator before using it and therefore

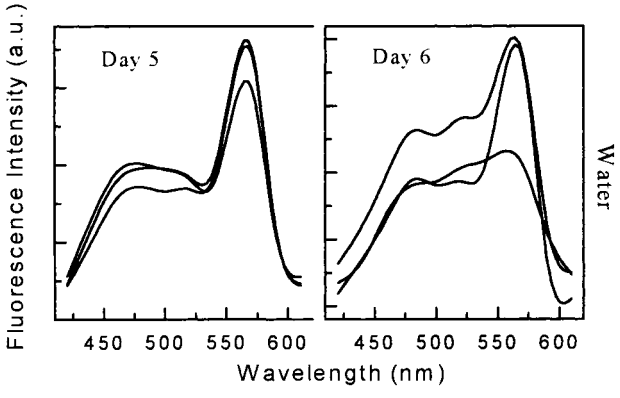

Fig. 7. Measured two-photon fluorescence spectra for group 1 muscle stored in water for 5 or 6 days.

offers the additional freedom of using the samples at a convenient time.

Handling and fixation are routine processes in most medical and biological research. It is required that the properties and the structures of samples remain as close to their original states as possible after excision and fixation, which means that the state of the microenvironment should be kept intact during the handling and the fixation processes. Fluorescence spectroscopy is one method that can be used to evaluate these processes because spectral information characterizes the states of the microenvironments within cells. The results shown in Subsections 3.A and 3.B indicate that the spectra of mouse skeletal muscle were characterized by two autofluorescence peaks. The changes in the spectra caused by the fixation, the handling, or both processes were identified by variations of these two fluorescence peaks. That the peak at wavelength 470 nm [NAD(P)H] varies more significantly than that at the wavelength of 540 nm (flavins) in methanol fixation addresses the mechanism involved in the process. Such analysis becomes possible under only two-photon excitation in which the two fluorescence peaks can be probed simultaneously.

4. Conclusion

In conclusion, the effects of the handling and the fixation processes on two-photon fluorescence spectroscopy of endogenous fluorophors in mouse skeletal muscle have been investigated. The two autofluorescence peaks excited by two-photon absorption vary differently according to different fixation and storage techniques. Formalin has a weak effect on the two-photon fluorescence spectra, whereas methanol reacts significantly to one of the two native fluorophors, NAD(P)H, and thus results in changes in its two-photon fluorescence spectra. Therefore formalin is a better fixative than methanol for preserving samples in *in vitro* research. Two-photon spectroscopy also shows no significant difference between the two different handling processes. However, the slice-sectioning process that uses the O.C.T. compound for prestorage offers an additional freedom in the sample-handing process and is recommended for noninvasive pathological research and clinical diagnosis for cancer detection under two-photon excitation.

The authors acknowledge support from the Australian Research Council (ARC) and the Victoria Breast Cancer Research Council for this project. Early research on this topic was conducted at Victoria University.

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