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Journal

LASERS IN MEDICAL SCIENCE, 13(3)

ISSN

0268-8921

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Publication Date

1998

DOI

10.1007/s101030050074

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Optical Biopsy of In Vivo Human Skin: Multi-photon Excitation Microscopy

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Abstract. Two-photon excitation microscopy has the potential as an effective, non-invasive, imaging tool for in vivo examination of human deep tissue structure at the subcellular level. By using infrared photons as the excitation source in two-photon microscopy, a significant improvement in penetration depth can be achieved because of the much lower tissue absorption coefficient and reduced scattering coefficient in the infrared wavelengths as compared to ultraviolet light. Two-photon absorption occurs primarily at the focal point and provides the physical basis for optical sectioning. Multi-photon excitation microscopy at 730 nm was used to image in vivo human skin autofluorescence from the surface to a depth of about 100 μm . The spectroscopic data suggest that reduced pyridine nucleotides, NAD(P)H, are the primary source of the skin autofluorescence using 730 nm excitation. This study demonstrates the use of multi-photon excitation microscopy for functional imaging of the metabolic states of in vivo human skin cells and provides a functional and morphological optical biopsy.

Keywords: Autofluorescence; Multi-photon; NADP(H); Redox imaging; Skin imaging; Two-photon

INTRODUCTION

A technique for functional and morphological optical biopsy of in vivo human skin is presented. NAD(P)H is an intrinsic probe of cellular metabolism and is a major source of in vivo human skin autofluorescence as characterised with fluorescence spectroscopy techniques [1–5]. Two-photon excitation microscopy with excitation at 730 nm can be used to monitor cellular metabolism, based on NAD(P)H fluorescence, in thick, highly scattering tissues such as in vivo human skin. Non-invasive measurements of tissue metabolism is finding applications in a number of important biomedical areas such as the diagnosis of cancer and the monitoring of wound healing. For cancer diagnosis, the change in NAD(P)H fluorescence has been linked to melanoma in skin and cancer in breast tissues [6–8]. Since the oxidative process is closely related to tissue health, the ability to image cellular NAD(P)H fluorescence in thick tissue may find applications in accessing the performance of various tissue

grafts in inducing wound healing or tissue regeneration [9,10].

Skin Anatomy

The skin is a complex, highly scattering tissue which is divided into three layers: the epidermis, the dermis and the subcutaneous tissue. The epidermis is the outermost portion of the skin and is composed of stratified squamous epithelium. The innermost layer of the epidermis consists of a single layer of cuboidal cells called basal cells. These cells differentiate and migrate towards the skin surface. The outer layer of the epidermis is called the stratum corneum which is composed of flattened and dead cells. The basal cells of the epidermis divide, differentiate and finally slough. As they migrate to the skin surface, the cells become more stratified and finally form the cornified layer of the stratum corneum.

Confocal Microscopy of In Vivo Human Skin

In order to perform optical biopsy it is necessary to optically section thick, highly scattering tissues such as in vivo human skin. A

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confocal microscope is an instrument designed to optically section thick specimens. Two apertures or pinholes are arranged in conjugate planes; one in front of the light source and one in front of the detector [11–13]. This arrangement results in improved optical lateral and axial resolution. It is the improved axial optical resolution that results in the enhanced optical sectioning capability.

In reflected light mode, confocal microscopes have been found to be very successful in imaging thick, highly scattering tissues. Reflected confocal systems have comparable penetration depth to a two-photon fluorescence system and provide complementary structural information. Reflected light microscopes visualise the biological interfaces where there are significant changes in the index of refraction, such as the plasma membrane and the nuclear envelope. This confocal principle has been exploited in a real-time, slit-scanning, confocal microscope that is used clinically for optical biopsy and diagnostics in ophthalmology [14]. The tandem-scanning confocal microscope, which operates in real-time, was developed to image optical sections of thick, highly scattering biological materials [15]. More recently, the tandem-scanning confocal microscope has been adapted for *in vivo* skin imaging [16–18]. Stacks of optical sections from *in vivo* skin that were acquired with the tandem-scanning reflected light confocal microscope can be visualised in three dimensions as orthogonal slices [19,20]. Another use of the reflected light scanning laser confocal microscope has been successfully developed for video rate imaging of *in vivo* skin [21]. However, the monitoring of cell metabolism in thick tissue using fluorescence confocal microscopes is less successful and this limitation is discussed in the following section.

Skin Autofluorescence

Cellular metabolism can be non-invasively optically monitored by the technique of redox fluorometry [1–5]. The probes are the intrinsic fluorescent pyridine nucleotides, and the flavoproteins. The pyridine nucleotides, NAD(P)H, are excited in the region 365 nm and fluoresce in the region 400–500 nm. The flavoproteins are excited in the region 450 nm and fluoresce in the region 500–600 nm. Typically, the fluorescence is imaged after ultraviolet excitation, and the changes in fluorescence intensity

follow changes in cell and tissue oxidative metabolism [5].

Metabolic imaging based on redox fluorimetry has two limitations. First, there is photodamage to the tissue and photobleaching of the fluorescent molecules along the excitation ultraviolet light path. Second, the use of short wavelengths precludes deep penetration of highly scattering tissues, such as human skin. It is necessary to perform spectroscopic studies to better characterise the intrinsic skin autofluorescence, since in addition to the NAD(P)H there are other sources of autofluorescence.

In preliminary studies of *in vivo* human skin autofluorescence, a laser scanning confocal microscope was used to image the natural autofluorescence induced by ultraviolet light (365 nm) and blue light (488 nm), and the fluorescence was detected at wavelengths longer than 515 nm. Optical sections of the stratum were obtained from the anterior surface of the index finger and the lower surface of the human forearm. Three-dimensional pseudo-colour depth-coded projections were formed from stacks of these optical sections [19]. This study has shown that metabolic diagnosis with one-photon confocal fluorescence microscope in highly scattering tissue is not feasible because the penetration depth of ultraviolet light needed for NAD(P)H excitation is less than 30 μm . Furthermore, the weak signal strength further prevents the source of the autofluorescence to be spectroscopically characterised.

Multi-photon Excitation Microscopy

Multi-photon excitation microscopy is an optical technique, limited to fluorescence imaging, that offers some advantages in the fluorescence imaging of highly scattering tissues such as *in vivo* human skin.

Maria Goeppert-Mayer developed the theoretical foundation for two-photon excitation processes in Göttingen, Germany in 1931 [22]. The physical principle is the simultaneous absorption of two photons; two photons of wavelength in the near infrared induce an electronic transition that normally requires an ultraviolet photon. The experimental verification of the two-photon absorption process is that the number of electronic transitions from the ground state to the excited states

is proportional to the square of the instantaneous intensity.

In general, fluorophores have two-photon absorption peaks at approximately two times their one-photon absorption wavelengths. However, this general finding should be experimentally verified for each fluorophore. In contrast to one-photon excitation, two-photon excitation can induce fluorescence emission at much shorter wavelengths. For example, two-photon excitation at 730 nm can excite UV chromophores such as NAD(P)H with absorption maximum on the order of 365 nm and eliminate the need to use ultraviolet excitation.

The use of a laser scanning microscope for fluorescence imaging based on two-photon excitation microscopy was first developed in 1990 [23]. The diffraction-limited focusing of the laser beam and the temporal concentration of the 100-fs pulses generated by the mode-locked laser result in two-photon excitation processes only in the region of focus. Outside the focal region there is insufficient photon density to induce any significant absorption. Therefore, there is little signal outside the focal region; this is the origin of the optical sectioning. The optical sectioning is derived from the non-linear optical response of the sample material and an aperture in front of the light detector is not required to obtain the optical sectioning capability of the microscope. In this way, a two-photon excitation microscope differs from a confocal microscope.

Before the development of two-photon excitation microscopy, in order to excite chromophores which absorb in the ultraviolet region, it was necessary to use ultraviolet excitation light. When ultraviolet fluorescence microscopy is used to image living cells and tissues, it was observed that cell viability was compromised and that there was considerable photobleaching of the chromophore.

The technique of two-photon excitation laser scanning microscopy has been used to mitigate the two problems cited previously; photobleaching and limited tissue penetration during imaging. The technique of two-photon excitation microscopy minimises the damaging effects of phototoxicity in living cells to the focal slice. This is very different from a conventional confocal microscope system in which the phototoxicity and photobleaching occur throughout the optical path. The first application of multi-photon excitation microscopy

in the study of thick tissue has been the three-dimensionally resolved NAD(P)H cellular metabolic imaging of an *in situ* cornea [24]. No emission spectra were measured in this study to characterise the primary fluorophores responsible for the observed fluorescence.

MATERIALS AND METHODS

Biological Sample

The lower surface of the right forearm was placed on the microscope stage. The measurement time was always less than 10 min. The estimated average power incident on the skin was 10–15 mW corresponding to peak powers between 500 and 1000 W. The photon flux incident on a diffraction limited spot on the skin is on the order of 10 MW/cm².

Multi-photon Scanning Microscope

A diagram of the multi-photon scanning microscope is shown in Fig. 1. This two-photon scanning microscope has been previously described [25,26]. A femto-second Ti:Sapphire laser (Mira 900, Coherent Inc., Palo Alto, CA) tuned to 730 nm is used as our light source. The average power entering the microscope is adjusted to 100 mW via a Glan-Thompson polariser. The microscope has a power transmission efficiency of 10%–20% resulting in 10–15 mW average power incident on the skin. The scanning is achieved via a galvanometer-driven x-y scanner (Cambridge Technology, Watertown, MA). The scan parameters are chosen such that 256 × 256 pixel images are generated with a pixel residence time of 40 μs corresponding to a frame rate of about 3 s. Occasionally, pixel residence time is increased to 400 μs to collect images with a higher signal to noise level. The excitation light enters the Zeiss Axiovert 35 microscope (Zeiss Inc., Thornwood, NY) via a modified epiluminescence light path. The light is reflected by the dichroic mirror to the objective. Two objectives were used in these experiments; a Zeiss 40 × Plan-Fluor (1.3 N.A., oil) is used for high resolution imaging and a Zeiss 20 × Plan-Neofluor (0.75 N.A., air) is used for its longer working distance into the tissue. The lateral resolution of these objectives is calibrated using known size fluorescent latex spheres. The 40 × objective has a lateral

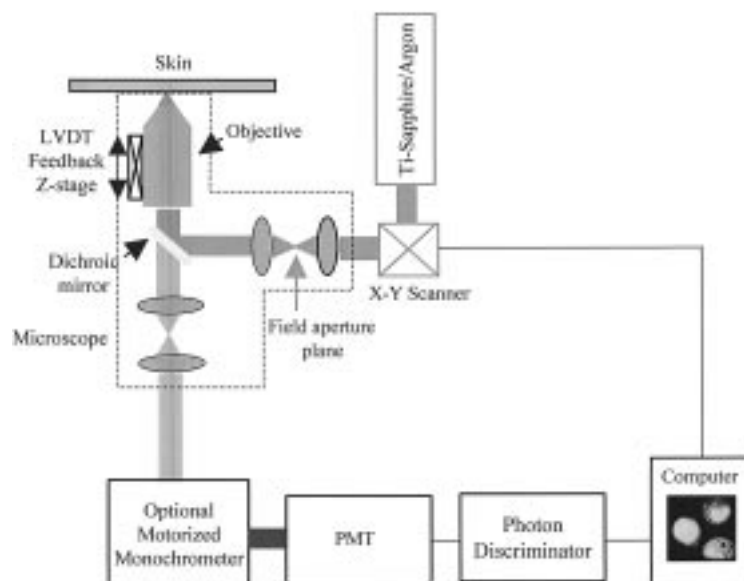


Fig. 1. Schematic of the two-photon scanning microscope with high sensitivity single photon counting electronics. PMT, photomultiplier tube; LVDZ, linear variable differential transformer; Ti:Sapphire/Argon, argon-ion laser pumped titanium-sapphire laser.

resolution of $0.20\ \mu\text{m}$ per pixel corresponding to an image size of $51.2\ \mu\text{m}$. The $20\times$ objective has a lateral resolution of $0.54\ \mu\text{m}$ per pixel corresponding to an image size of $137\ \mu\text{m}$. The axial resolution of the $40\times$ objective is on the order of $1\ \mu\text{m}$ and the $20\times$ objective is on the order of $3\ \mu\text{m}$. The axial resolution of this imaging system is determined under ideal conditions where the sample has minimal scattering. In the tissue, multiple scattering is expected to degrade the two-photon point spread function. The determination of two-photon point spread function as a function of tissue parameters such as scattering and absorption coefficient is a major focus of current research. The fluorescence signal is collected by the same objective, transmitted through the dichroic mirror and barrier filter (3 mm BG-39, CVI Laser, CA) and re-focused on the detector. The fluorescence signal at each pixel is detected by a photomultiplier tube (PMT). For intensity measurements, a low noise, single photon counting R5600-P PMT or a high gain cooled R1104 PMT (Hamamatsu, Bridgewater, NJ) is used for single photon counting. The output of the PMT is converted into single photoelectron pulses via a low noise amplifier/discriminator (Pacific AD6, Concord, CA). The number of photons collected at each pixel is counted and digitally recorded through a custom-built electronic interfaced card by the control computer. Custom software synchronises this data acqui-

sition process with the scanner movement to generate a two-dimensional image. A three-dimensional imaged stack can be obtained by controlling the microscope objective height with a stepper motor; the microscope objective position is monitored using a linear variable differential transformer (Schaevitz Engineering, Camden, NJ). An image stack with depth up to $200\ \mu\text{m}$ into the skin has been obtained. The typical depth increment used in these experiments was $5\ \mu\text{m}$. The image stack was stored digitally. Three-dimensional reconstruction and presentation were performed using Spyglass Slicer Software (Fortner Research, Sterling, VA).

Measurement of the Fluorescence Emission Spectrum in the Microscope

Measurements of the skin emission spectrum were performed at selected points. A motor-driven monochromator is inserted between the microscope and the photomultiplier tube. The monochromator is driven at a constant speed of $1\ \text{nm/s}$. The wavelength resolution is set by the monochromator slit to $2\ \text{nm}$. The x-y scanner is set to remain at the origin during spectral measurement. Data were collected in photon counting mode. The fluorescence spectrum is measured at an excitation wavelength of $730\ \text{nm}$. Spectra were taken at two

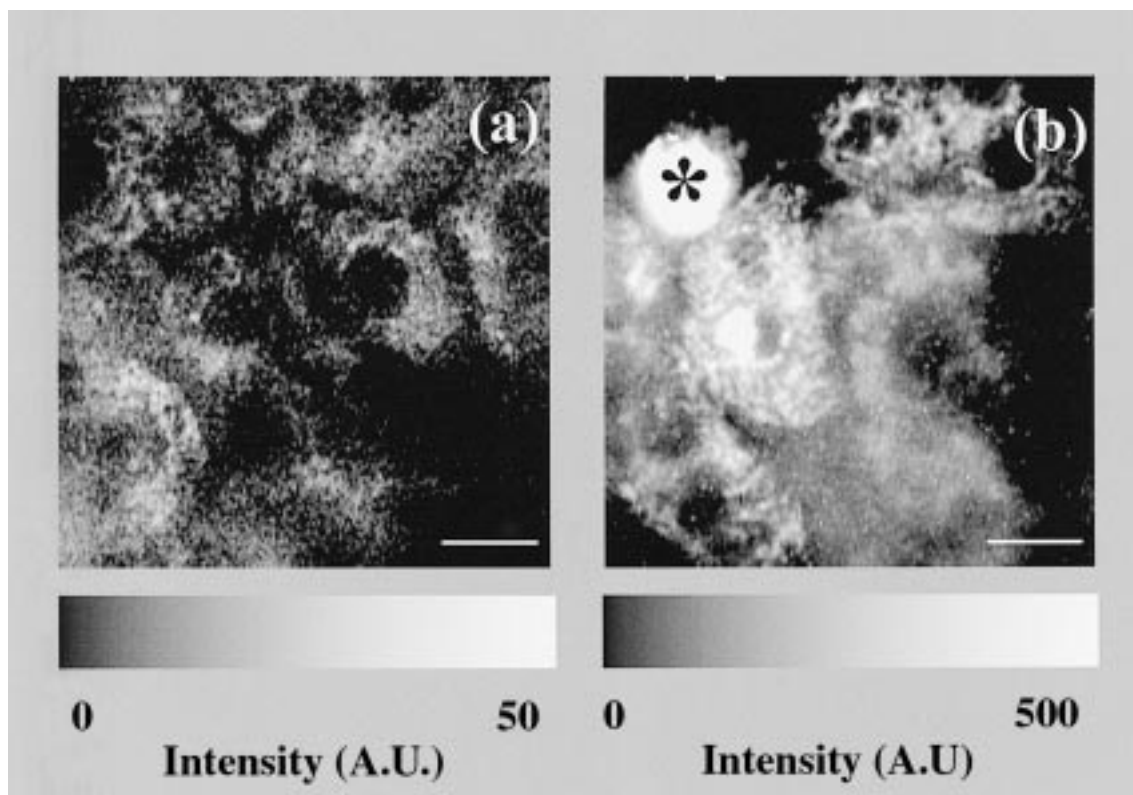


Fig. 2. Representative images of epidermal cell layers obtained with multi-photon excitation microscopy using an excitation wavelength of 730 nm. (a) An image of a cell layer collected at a frame rate of 3 s. (b) An image of a different cell layer collected at a frame rate of 30 s. Scale bar=10 μm . The relative pixel intensity in the images is represented by the grey scale maps below the images. The average intensity of (b) is ten times greater than the average intensity of (a). In the longer exposure image, (b), the punctate intensity pattern observed is consistent with cytoplasmic mitochondria distribution. The high intensity region indicated by the asterisk extends over many cell layers and may represent extracellular fluorescence material. The intensity in this region is over an order of magnitude beyond the maximum of the grey scale map and is, therefore, truncated.

depths: the surface (0–50 μm) and below the epidermis (100–150 μm).

RESULTS

Multi-photon Optical Biopsy of In Vivo Human Skin

We observed individual cells within the thickness of the skin at depths from 25 to 75 μm below the skin surface. No cells were observed within the stratum corneum. This is consistent with studies using reflected light confocal microscopy [20]. Cellular fluorescence originates from the cytoplasm. This is consistent with the assumption that NAD(P)H provides the major contribution to the cellular autofluorescence observed. The cell nuclei and the cell borders appear dark due to the absence of fluorescence. Cells of 15–20 μm diameter were imaged at an approximate depth of 40–50 μm . Cells of about 8–10 μm diameter were

observed at an average depth of 25–30 μm . Within the cytoplasm of the cells, punctated fluorescence can be observed. This is consistent with a previous study of isolated single cells and these fluorescent organelles are likely to be mitochondria with a high concentration of NAD(P)H [27]. The fluorescence signal decreased for deeper images as expected from the exponential decrease of light transmission in a highly scattering medium. Individual cells observed at depths from 40 to 80 μm are shown in Fig. 2. These results are consistent with the cellular structures observed in the stratum spinosum by confocal microscopy and histological studies. Intensity variation is observed below 80 μm but no structures can be discerned.

Figure 3 shows the three-dimensional representation of the NAD(P)H autofluorescence with two-photon excitation 730 nm. There are two regions of high fluorescence; the layers near the skin surface, and the layers that correspond to the basal cells at the top of the

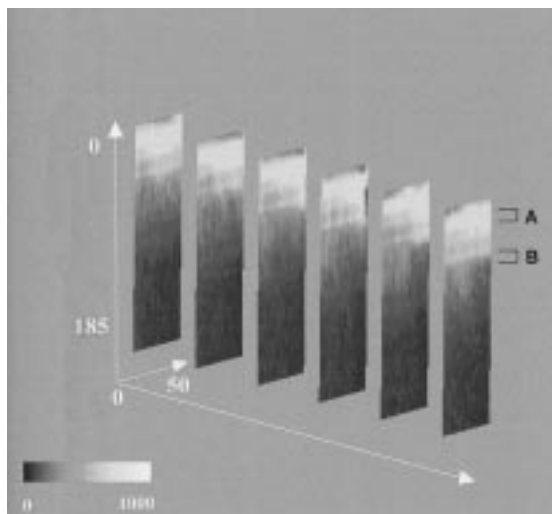


Fig. 3. Two-photon excited three-dimensional image of in vivo human skin at 730 nm excitation. The reconstructed 3-D image of human skin is presented as a set of X–Z cross-section slices. The units of the axis dimensions are in μm . The individual X–Z slices are separated by $8 \mu\text{m}$. The top bright layer (A) corresponds to the stratum corneum located at the skin surface. The second bright band (B) at a depth of $50\text{--}100 \mu\text{m}$ is the basal cell layer at the top of the papillary dermis.

papillary dermis. The deeper fluorescent band corresponds to the basal cell layer. Since NAD(P)H production is correlated with cellular redox activity, the higher fluorescence from the basal layer reflects the enhanced metabolism of this layer of highly active cells. The fluorescence band close to the surface corresponds to the stratum corneum but the origin of its fluorescence is still under investigation. One conjecture is that the stratum corneum comprises many layers of stratified squamous epithelium and thus contains a much higher density of NAD(P)H.

Spectroscopic Characterisation of the Emission Spectra

Since many tissue components, both cellular and extracellular, are fluorescent, the major autofluorescent species in most tissues are poorly characterised. The better known fluorescence species include NAD(P)H flavoproteins, collagen and elastin. Better spectroscopic study will allow tissue fluorescent structures to be identified. Moreover, it may also provide functional information of the underlying biochemical driving forces in tissues. At selected points on the skin, fluorescent spectra were measured close to the

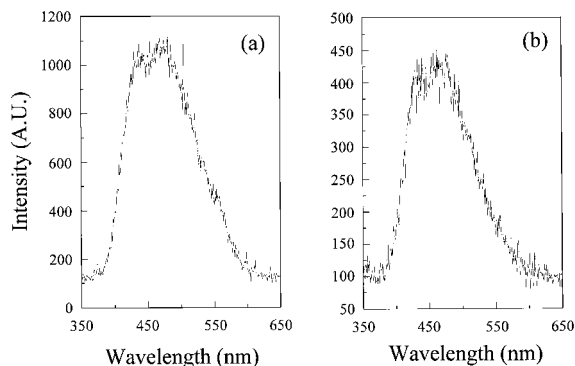


Fig. 4. Two-photon excited fluorescence emission spectra (730 nm) of in vivo human skin. This excitation wavelength roughly corresponds to a one-photon absorption of 365 nm. Intensity is given in photon counts per second. Representative spectra were presented from data taken (a) at the surface region between 0 and $50 \mu\text{m}$ deep and (b) at a depth between 100 and $150 \mu\text{m}$.

stratum corneum ($0\text{--}50 \mu\text{m}$) and deep inside the dermis ($100\text{--}150 \mu\text{m}$). Two-photon excitation experiments were performed for 730 nm wavelength which corresponds to a single photon excitation of approximately 365 nm. Figure 4(a) shows a typical fluorescent spectrum obtained at different points in the $0\text{--}50 \mu\text{m}$ skin layer excited with 730 nm excitation. The whole emission band is wide covering a region from 400 to 600 nm. At deeper regions ($100\text{--}150 \mu\text{m}$) the same spectrum is observed with some decrease in fluorescence intensity as expected from the multiple scattering medium. The observation of fluorescence emission at shorter wavelengths indicates that the fluorescence originated from multi-photon processes. The spectrum observed at $100\text{--}150 \mu\text{m}$ deep into the dermis is presented in Fig. 4(b).

In summary, the fluorescence emission spectroscopy results are consistent with the following. Two-photon excitation at 730 nm shows an emission spectrum consistent with NAD(P)H fluorescence at both the surface of the skin and at deeper levels up to $150 \mu\text{m}$ below the skin surface.

DISCUSSION

Two-photon imaging is particularly suitable for studying deep tissue. The near-infrared light used in the two-photon microscope can penetrate deeper in highly scattering tissues such as in vivo human skin than confocal microscopes operated with ultraviolet excitation. In addition, the two-photon light collection efficiency is higher than that

obtainable with a standard point scanning confocal microscope since two-photon excitation microscopy uses neither a pinhole nor descanning optics. The optical sectioning is originated in the non-linear absorption process. Therefore, a large area detector can be used to maximise the collection efficiency of the fluorescence photons which may be scattered as they travel through the turbid sample. In terms of tissue photodamage, the near-infrared radiation used in two-photon excitation is less phototoxic than ultraviolet radiation. The localisation of the two-photon excitation volume to the focal plane further minimises the volume where tissue damage may occur.

We have demonstrated the use of multi-photon excitation microscopy at 730 nm to image the autofluorescence of in vivo human skin from the surface to a depth of about 100 μm . The recording of emission spectra further enables the characterisation of the source of the autofluorescence. Our results support NAD(P)H as the primary source of the autofluorescence at 730 nm excitation. Multi-photon excitation opens the possibility of an optical biopsy of in vivo human skin based on functional imaging of cellular metabolites, and therefore may have diagnostic potential in dermatology and the cell biology of in vivo human skin.

ACKNOWLEDGEMENTS

BRM acknowledges support from NIH EY-06958 (BRM). PTS acknowledges support from America Cancer Society (RPG-98-058-01-CCE), NSF (MCB-9604382), NIH (R55GM/OD56486-01), DuPont Educational Aid Program and the Center for Bioengineering, MIT. EG and The Laboratory for Fluorescence Dynamics, Department of Physics is supported by the National Institute of Health (RR03155).

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*Received for publication 22 July 1997;
accepted following revision 23 February 1998.*