Title

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Two-Photon Optical Biopsy Of Thick Tissues

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Abstract

Two-photon optical biopsy has been performed in human and mouse skin. Both cellular and extracellular tissue components have been visualized based on endogenous fluorescence.

Introduction

An assessment of tissue physiological states requires morphological information with sub-cellular details. This sub-cellular structural information is traditionally acquired from histological sections of excised tissue. Much of the cellular biochemical information is lost during the surgical and fixation processes. More importantly, the biopsy procedure is clinically undesirable due to its invasive nature. The development of a non-invasive optical technology that can acquire cellular structural and biochemical signals from within deep tissue will bring major advances in clinical diagnosis technology.

The opacity of typical tissues is a major obstacle in non-invasive optical tissue diagnosis. Today, one of the most promising approaches is two-photon excitation microscopy introduced by Denk et. al. [1]. Fluorophores can be excited by the simultaneous absorption of two photons each having half the energy needed for the excitation transition. Since the two-photon excitation probability is significantly less than the one-photon probability, appreciable two-photon excitation occurs only at the focal point, a region of high temporal and spatial concentration of photons. Two-photon excitation allows 3-D tissue structures to be imaged with resolution comparable to confocal microscopes but with a number of significant advantages when the sample's absorption and scattering coefficients are high: (1) The typical scattering and absorption coefficients in the infrared spectral range is over an order of magnitude less than the near UV or the blue-green region. The use of infrared excitation in the two-photon microscope minimizes the attenuation of the excitation signal. (2) Confocal microscopy uses the emission pinhole aperture to reject out of focus light. Inside deep tissue, scattering of the signal photons is inevitable. The consequent path deviation results in a significant loss of these photons at the confocal pinhole. The collection geometry for the fluorescence photons is less critical in the two-photon case where a large area detector can be used without a pinhole aperture. Most of the forward scattered photons can be retained. (3) Two-photon excitation minimizes tissue photo-damage. Conventional confocal techniques obtain 3-D resolution by limiting the observation volume, but fluorescence excitation occurs throughout the hourglass-shaped light path. In contrast, two-photon excitation limits the region of photo-interaction to a sub-femtoliter volume at the focal point.

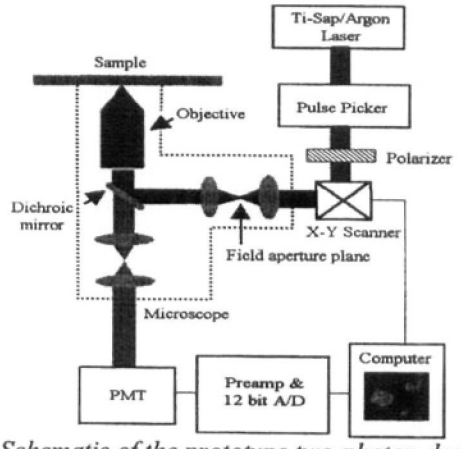

Fig. 1. Schematic of the prototype two-photon deep tissue microscope

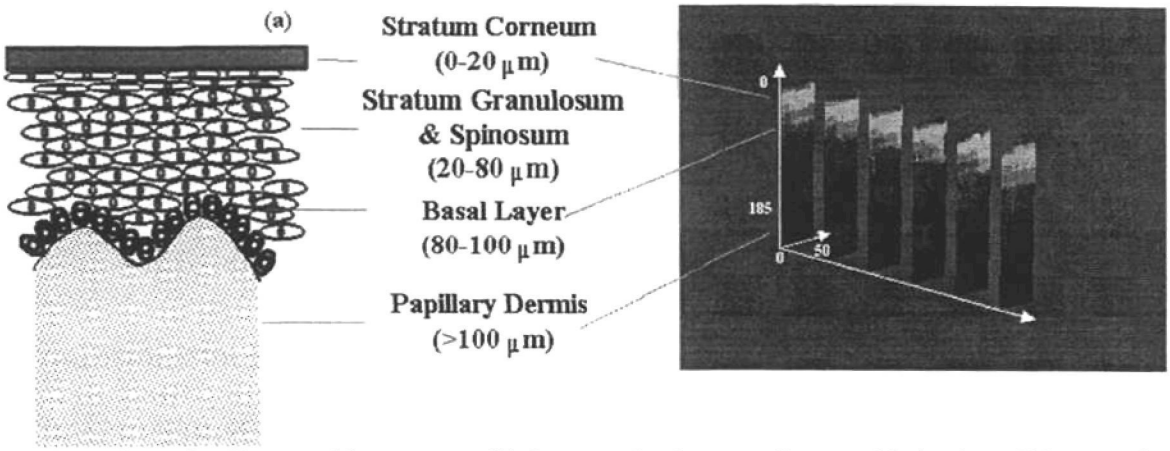

Figure 2. (a) A schematic of human skin anatomy. (b) Cross section images of human skin in vivo: 730 nm excitation. X, and Y orthogonal slices are shown. Units are shown in μm.

For non-invasive two-photon tissue biopsy, fluorescent labeling with extrinsic probe is undesirable and is often difficult. Fortunately, many tissue components are autofluorescent. Endogenous cellular proteins such as β-nicotinamide-adenine dinucleotide phosphate (NAD(P)H) and a number of flavoproteins are fluorescent [2,3]. Extracellular matrix components such as collagen [4] and elastin [5] can also be excited using UV excitation. Unfortunately, many of these biological molecules are poor fluorophores with low extinction coefficient and quantum efficiency. Therefore, successful non-invasive two-photon imaging of deep tissue structures requires careful optimization of detection optics.

Experimental Methods

A deep tissue microscope based on two-photon excitation has been constructed (Fig. 1). The laser used in this microscope is a mode-locked Titanium-Sapphire laser (Mira 900, Coherent Inc., Palo Alto, CA). The beam expanded laser light is directed into the microscope via a galvanometer-driven x-y scanner (Cambridge Technology, Watertown, MA). Images are generated by raster scanning the x-y mirrors. The excitation light enters the Zeiss Axiovert 110 microscope (Zeiss Inc., Thronwood, NY) via a modified epi-luminesence light path. The scan lens is positioned such that the x-y scanner is at its eye-point while the field aperture plane is at its focal point. Since the objectives are infinity-corrected, a tube lens is positioned to re-collimate the excitation light. The scan lens and the tube lens function together as a beam expander which over-fills the back aperture of the objective lens. The dichroic mirrors are custom made short pass filters (Chroma Technology Inc., Brattleboro, VT) which maximize reflection in the infrared and transmission in the blue-green region of the spectrum. The objective used in most of these studies is a Zeiss Fluar 40X with numerical aperture of 1.25. This objective is chosen for its high numerical aperture and its high throughput. The high numerical aperture is essential for the formation of a diffraction limited spot on the order of 0.1 femtoliter. The objective axial position is controlled by a piezo-stage (PI Inc., Auburn, MA). The axial resolution of this system is about 1 micron with a range of over 100 micron. The microscope field of view is about 50-200 μm on a side and the typical frame rate is about two seconds. The fluorescence emission is collected by the same objective and transmitted through the dichroic mirror along the emission path. An additional barrier filter is needed to further attenuate the scattered excitation light because of the high excitation intensity used. A de-scan lens is inserted between the tube lens and the photomultiplier tube (PMT). The number of available fluorescence photons is always a limiting factor in non-invasive deep tissue imaging. On one hand, the scattering and absorption properties of thick tissue quickly diminishes both the excitation and the emission light. On the other hand, we always seek to use the lowest excitation power possible to limit photodamage to the tissue. In this low light situation, we have implemented a single photon counting signal detection system. The fluorescence signal at each pixel is detected by a R5600-P PMT (Hamamatsu, Bridgewater, NJ) which is a compact single photon counting module with high quantum efficency of 12% at 500 nm and 25% at 400 nm. The dark noise of this PMT is less than twenty photon counts per second at room temperature. This high signal to noise ratio is crucial for high sensitivity detection. A 100 MHz single photon counting discriminator (F-100T, Advanced Research Instruments, Boulder, CO) converts single photon bursts into TTL pulses. The number of collected photons are counted using a home built interface circuit and the number is transferred to the data acquisition computer.

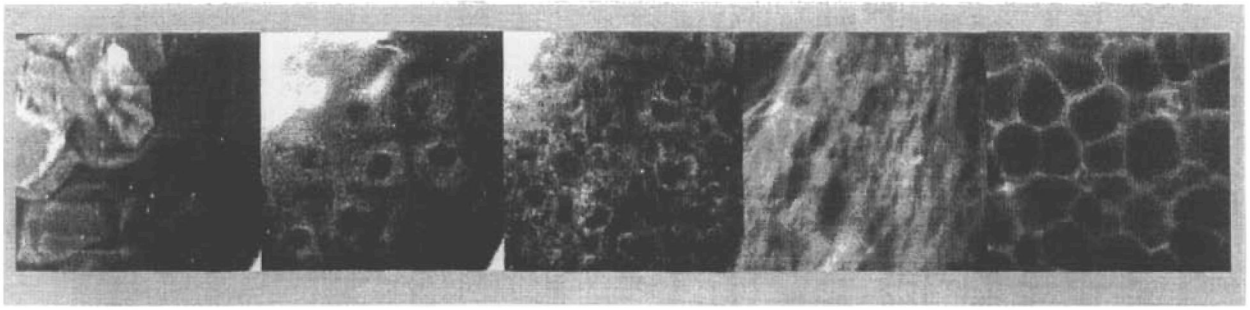

Fig. 3: Mouse ear dermal structure (from left to right): stratum corneum (0 μm), epidermal cell layer (10 μm), basal cell layer (15 μm), dermal layer (30 μm), dermal layer (50 μm).

Results

Cellular structural information has been obtained from in vivo human skin at the lower surface of the forearm [6]. Two-photon microscopy imaging was accomplished by exciting cellular auto-fluorescence from endogenous chromophores which primarily consist of NAD(P)H. The production of NAD(P)H is associated with the cellular metabolism and the intracellular redox state. Typically, a higher metabolic rate generates a higher concentration of NAD(P)H and produces brighter images. For these experiments, the Ti-Sapphire laser is tuned to 730 nm. The power incident upon the skin surface ranges from 10 to 20 mW. Z-section images were taken at depths of every five microns. Three dimensional images were reconstructed using Spyglass Slicer software. Fig. 2b displays the x-z planes of the reconstructed volume. The brightly fluorescent layer at the top corresponds to the stratum corneum. The second bright layer about 100 μm below the skin's surface has an "egg carton" like morphology which most likely corresponds to the basal cell layer at the dermal-epidermal junction. The higher fluorescence from this layer correlates well with the high activity level of the basal cells. Fig. 2a shows a schematic of the skin structure as confirmed by either confocal or electron microscopy; a good correlation between the two-photon results and the known skin physiology is observed.

Mouse skin structure at the tip of its ears has also been visualized using two-photon microscopy. The imaging was performed within four hours of the euthanasia procedure. In addition to visualizing cellular distribution in the epidermal layer, we have succeeded in imaging collagen/elastin like structures in the dermal layer. Fluorescence images of mouse epidermal/dermal structures are shown in Fig. 3.

Conclusion

We have demonstrated the feasibility of imaging deep tissue structures from a mouse model and an in vivo human subject. Most importantly, we have demonstrated that endogenous chromophores can provide sufficient contrast for the visualization of deep tissue structures. In particular, cellular morphology in deep tissue can be imaged based on NAD(P)H cytoplasmic fluorescence. The extracellular matrix structure can be mapped based on collagen or elastin autofluorescence. The ability to simultaneously visualize cellular and extracellular matrix structures in tissues is critical for the understanding of biomedical processes such as carcinogensis and tissue regeneration.

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