Confocal microscopy and multi-photon excitation microscopy of human skin in vivo

Barry R. Masters
Department of Ophthalmology, University of Bern, Bern, 3010 Switzerland
barrymas@bicwater.usuf1.usuhs.mil

Peter T. C. So
Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139 USA
ptso@mit.edu

Abstract: This paper compares tandem scanning reflected light confocal microscopy and multi-photon excitation microscopy for the observation of human skin in vivo. Tandem scanning confocal light microscopy based on a white light source can provide video-rate image acquisition from the skin surface to the epidermal-dermal junction. Multi-photon excitation is induced by a 80 MHz pulse train of femtosecond laser pulses at 780 nm wavelength. This nonlinear microscopic technique is inherently suitable for deep tissue fluorescence imaging. The relative merits of these two techniques can be identified by comparing movies of optical sections obtained from the forearm skin of the same volunteer.

OCIS codes: (180.6900) Three-dimensional microscopy; (170.1790) Confocal microscopy; (190.4180) Multiphoton processes

References and links

1. B. R. Masters, P. T. C. So, and E. Gratton, "Optical biopsy of in vivo human skin: multiphoton excitation microscopy," Lasers Med Sci **13**,196-203 (1998).
2. C. Buehler, K. H. Kim, C. Y. Dong, B. R. Masters and P. T. C. So, "Innovations in two-photon deep tissue microscopy," IEEE Engineering in Medicine and Biology **18**, 23-29 (1999).
3. B. R. Masters, P. T. C. So, K. H. Kim, C. Buehler, and E. Gratton, "Multiphoton excitation microscopy, confocal microscopy, and spectroscopy of living cells and tissues: functional metabolic imaging of human skin in vivo," in *Methods in Enzymology, Confocal Microscopy*, vol. 307, P. M. Conn, ed. (Academic Press, New York, 1999) Chap. 29.
4. P. Corcuff, G. Gonnord, G. E. Pierard, and J. L. Leveque, "In vivo confocal microscopy of human skin: a new design for cosmetology and dermatology." Scanning **18**, 351-355 (1996).
5. P. Corcuff and J. L. Leveque, "In vivo vision of the human skin with the tandem scanning microscope." Dermatology **186**, 50-54 (1993).
6. P. Corcuff, C. Bertrand and J. L. Leveque, "Morphometry of human epidermis in vivo by real-time confocal microscopy." Arch Dermatol Res. **285**, 475-481(1993).
7. M. Rajadhyaksha, M. Grossman, D. Esterowitz, R. H. Webb and R. Anderson, "In vivo confocal scanning laser microscopy of human skin: melanin provides strong contrast." J. Invest Dermatol **104**, 946-952 (1995).
8. B. R. Masters, "Three-dimensional confocal microscopy of human skin in vivo: autofluorescence of normal skin." Bioimages **4**, 13-19 (1996).
9. B. R. Masters, D. J. Aziz, A. F. Gmitro, J. H. Kerr, T. C. O'Grady and L. Goldman, "Rapid observation of unfixed, unstained, human skin biopsy specimens with confocal microscopy and visualization." J. Biomed. Optics, 2,437-445 (1997).
10. B. R. Masters, G. Gonnord and P. Corcuff, "Three-dimensional microscopic biopsy of in vivo human skin: a new technique based on a flexible confocal microscope." J. Micros. **185**, 329-338 (1997).
11. C. Bertrand and P. Corcuff, "In vivo spatio-temporal visualization of the human skin by real-time confocal microscopy," Scanning **16**, 150-154 (1994).
12. B. R. Masters and P. T. C. So, "Multi-photon excitation microscopy and confocal microscopy imaging of in vivo human skin: a comparison," Micros. Microanal. **5**, 282-289 (1999).
13. R. R. Anderson and J. A. Parrish, "The optics of human skin." J. Invest Dermatol **77**,13-19 (1981).
14. W. J. Denk, J. P. Strickler and W. W. Webb, "Two-photon laser scanning fluorescence microscopy." Science **248**, 73-76 (1990).

#27001 - $15.00 US Received October 20, 2000; Revised December 05, 2000

15. W. J. Denk, D. W. Piston and W. W. Webb, *Handbook of Biological Confocal Microscopy*, J.B. Pawley, ed. (Plenum Press, New York, 1995).
16. W . Denk, "Two-photon excitation in functional biological imaging." J. Biomed. Optics **1**, 296-304(1996).
17. W. J. Denk , J. P. Strickler and W. W. Webb, "Two-photon laser microscopy." United States Patent, 5,034,613, July 23, 1991.
18. P. T. C. So, C. Y. Chen, B. R. Masters, and K. M. Berland, "Two-photon excitation fluorescence microscopy," in *Ann. Rev. Biomedical Engineering*, M.L. Yarmush, K. R. Diller, M. Toner ed. (Annual Reviews, Palo Alto, CA 2000).
19. K. Konig, P. T. C. So , W. W. Mantulin and E. Gratton, "Cellular response to near-infrared femtosecond laser pulses in two-photon microscopes."Opt. Lett. **22**, 135-136 (1997).
20. K. Konig, P. T. C. So , W. W. Mantulin, B. J. Tromberg and E. Gratton, "Two-photon excited lifetime imaging of autofluorescence in cells during UVA and NIR photostress." J. Micros. **183**, 197-204(1996).
21. T. Wilson, *Confocal Microscopy*, (Academic Press, London 1990).
22. M. Gu, *Principles of three-dimensional imaging in confocal microscopy*, (World Scientific, Singapore 1996).
23. T. R. Corle and G. S. Kino, *Confocal scanning optical microscopy and related imaging systems* (Academic Press, San Diego 1996).
24. J. B. Pawley, *Handbook of Biological Confocal Microscopy*, (Plenum Press, New York, 1995).
25. B. R. Masters, *Selected Papers on Confocal Microscopy*, (SPIE Press, Bellingham, WA, 1996).
26. P. T. C. So, T. French, W. M. Yu, K. M. Berland, C. Y. Dong and E. Gratton, "Time-resolved fluorescence microscopy using two-photon excitation." Bioimaging **3**, 49-63 (1995).
27. P. T. C. So, H. Kim, I. E. Kochevar, "Two-photon deep tissue ex vivo imaging of mouse dermal and subcutaneous structures." Optics Express, **3**, 339-350 (1998). http://www.opticsexpress.org
28. B . Chance, "Pyridine nucleotide as an indicator of the oxygen requirements for energy-linked functions of mitochondria." Circ. Res. Suppl. 1, **38**,I-31 - I-38 (1976).
29. D. W. Piston, B. R. Masters and W. W. Webb, "Three-dimensionally resolved NAD(P)H cellular metabolic redox imaging of the in situ cornea with two-photon excitation laser scanning microscopy." J. Micros. **178**, 20-27 (1995).
30. K-H. Kim, C. Buehler, and P. T. C. So, "High-speed two-photon scanning microscope," Appl. Opt. **38**, 6004-6009 (1999).
31. J. Bewersdorf, R. Pick and S. W. Hell, "Mulitfocal multiphoton microscopy," Opt. Lett. **23**, 655-657 (1998).
32. G. J. Brakenhoff, J. Squier, T. Norris, A. C. Bliton, W. H. Wade and B. Athey "Real-time two-photon confocal microscopy using a femtosecond, amplified Ti:sapphire system," J Microsc. **181**, 253-259 (1996).

1. Introduction

We have compared the use of multi-photon excitation microscopy [1-3] and reflected light tandem scanning microscopy [4-11] for imaging human skin in vivo. The studies were performed on the similar fields of the same human subject; therefore, the observed images are due to differences of the two microscopic techniques [12]. For observation of human skin in vivo both techniques gave similar results from the skin surface to the epidermal-dermal junction. Multi-photon excitation microscopy resolved the individual squames within the stratum corneum and in general showed enhanced contrast. For observation of the dermis the ability of the multi-photon excitation microscope to image the collagen and elastin fibers is a significant advantage.

2. Structure of human skin

Adult skin is a dynamic tissue [13]. The outer layers of the epidermis form the stratum corneum; cornified dead cells are in the process of being sloughed off. The inner layer of cells in the basal cell layer are in the process of proliferation and differentiating which forms new cells at the surface. The next layer of polyhedral keratinocytes forms the stratum spinosum. Adjacent to the stratum spinosum is the stratum granulosum. The surface layers consist of five or six layers of flattened squamous cells that are devoid of nuclei. The layers of the skin are schematically shown in Figure 1. Four types of cells are located within the living epidermis: keratinocytes, and a few percent of dendritic cells: Langerhans cells, melanocytes, and rare Merkel cells. Melanocytes which are pigment cells form the brown pigment melanin which is formed into vesicles called melanosomes. Eventually the melanosomes are transferred from the melanocytes into the basal epithelial cells. The epidermis also contains other structures

including: free nerve endings, hair, sweat and apocrine glands. The dermis is located under the epidermis and primarily consists of connective tissue (elastin and collagen fibers).

3. Multiphoton excitation microscopy

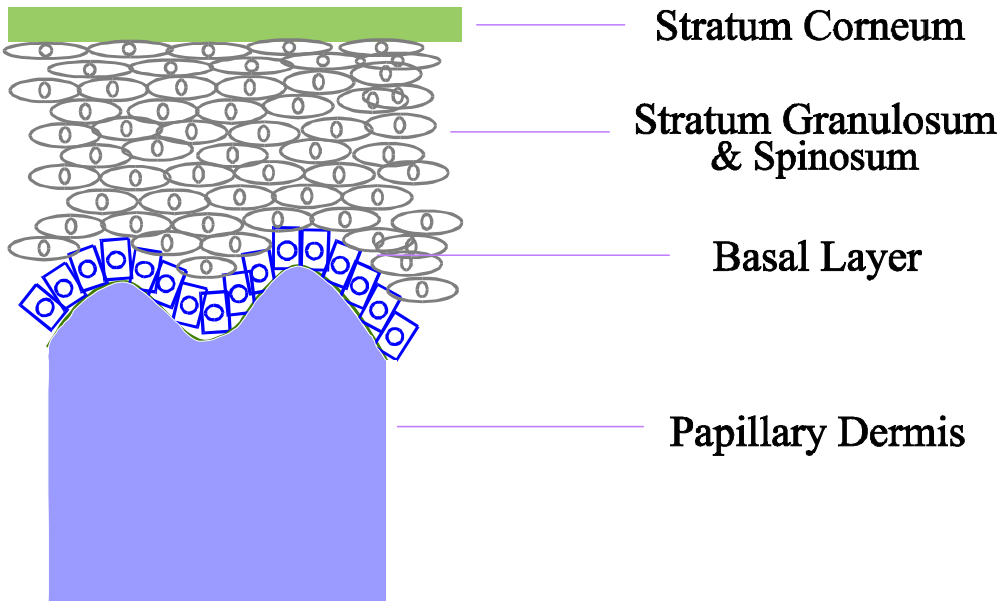

Fig. 1. Schematic drawing of a vertical section of human skin. The following layers are shown: stratum corneum, stratum granulosum, stratum spinosum, the basal layer, and the papillary dermis.

Multi-photon excitation processes were investigated for several decades. The important work of Denk, Strickler and Webb which was published in Science in 1990 launched a new revolution in nonlinear optical microscopy [14-18]. They implemented multi-photon excitation processes into microscopy by integrating a laser scanning microscope (scanning mirrors, photomultiplier tube detection system) and a mode-locked laser which generates pulses of near-infrared light. The pulses of red or near-infrared light (700 nm) were less than 100 fsec in duration and the laser repetition rate was about 80 MHz. These pulse have sufficiently high peak power to achieve two-photon excitation at reasonable rates at an average power less than 25 mW, that induces minimal photodamage to many types of biological samples [19,20]. However, highly pigmented cells and tissues could be subjected to photo-induced thermal damage. The potential benefits of two-photon excitation microscopy include: reduced photobleaching of the fluorophores, improved background discrimination, and minimal the photodamage to living cell specimens.

In a two-photon excitation process, the rate of excitation is proportional to the average squared photon density. This quadratic dependence follows from the requirement the fluorophore must simultaneously absorb two photons per excitation process. The laser light in a two-photon excitation microscope is focused by the microscope objective to a diffraction limited focal volume. Only in this focused volume is there sufficient intensity to generate appreciable excitation. The low photon flux outside the focal volume results in a negligible amount of fluorescence signal. The origin of the optical sectioning capability of a two-photon

#27001 - $15.00 US Received October 20, 2000; Revised December 05, 2000

excitation microscope is due to the nonlinear quadratic dependence of the excitation process and the strong focusing capability of the microscope objective. The focusing of the microscope objective results in two-photon excitation of ultraviolet absorbing fluorochromes in a small focal volume. It is possible to move the focused volume through the thickness of the sample and thus achieve optical sectioning in three-dimensions. The optical sectioning in a two-photon excitation microscope occurs during the excitation process. The emitted fluorescence can then be detected, without the requirement of descanning, by placing an external photon detection device as close as possible to the sample.

4. Confocal Microscopy

There are a number of books and articles which describe the principles and applications of confocal microscopy [21-24]. A history of the confocal microscope from the 1884 patent of Nipkow to the present is found in a compilation of selected milestone papers which have been reprinted in a book [25].

A confocal microscope illuminates and detects the scattered or fluorescent light from the same volume within the specimen. A set of conjugate apertures, one for illumination and one for detection of the scattered or fluorescent light function as spatial filters. There are two enhancements of the imaging characteristics of a confocal microscope as compared with standard light microscopes: (1) enhanced lateral resolution, and (2) enhanced axial resolution. It is the latter effect which lends itself to the optical sectioning of thick specimens. The increased axial resolution results in a strong rejection of light from regions outside of the focal plane. The main advantage of a confocal microscope is its ability to optically section thick specimens. Contrast is provided from differential scattering due to differences in refractive index between various cellular and extracellular features in the field. The microscope objective of a confocal microscope illuminates the specimen with a double inverted cone of light. Photobleaching of fluorophores occurs in the entire inverted illumination cone of light as well as in the focal volume.

5. Microscopy of human skin in vivo: multi-photon excitation microscopy

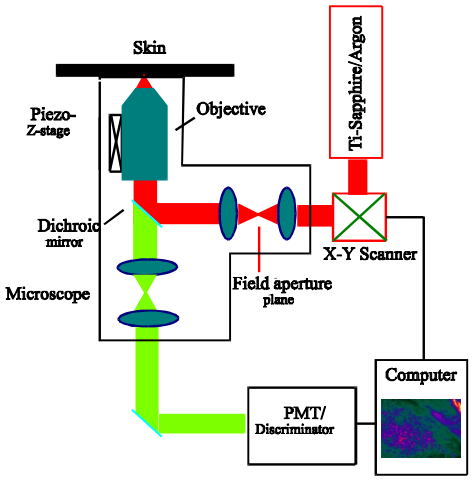

Fig. 2. Schematic of a two-photon deep tissue microscope.

The instrumentation and design of a basic multi-photon microscope has been described in a several previous publications [26,27]. **Figure 2** illustrates the key components of a multi-photon excitation microscope. The multi-photon excitation microscope design is based on a

#27001 - $15.00 US
Received October 20, 2000; Revised December 05, 2000

mode-locked Titanium-Sapphire laser (Mira 900, Coherent Inc., Palo Alto, CA). A Glan-Thomson polarizer and a laser pulse picker, are used either alone or in combination to control the excitation laser pulse train profile. 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 microscope (Zeiss Inc., Thornwood, NY) via a modified epi-luminescence 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 reflects the excitation light to the objective. 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 microscope objective used was a Zeiss Plan-Neofluar multi-immersion objective (25X, 0.8 N.A.). Typical image size is on the order of 200 microns on a side. The objective axial position is driven by a piezo-motor interfaced to a computer. The typical image acquisition time 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. Since two-photon excitation has the advantage that the excitation and emission wavelengths are well separated (by 300-400 nm), short pass filters such as 2 mm of BG39 Schott glass filter (CVI Laser, Livermore, CA) eliminate most of the residual scatter with a minimum attenuation of the fluorescence. A de-scan lens is inserted between the tube lens and the photomultiplier tube (PMT). The de-scan lens re-collimates the excitation. It also ensures that the emission light strikes the PMT at the same position that is independent of scanner motion. A single photon counting signal detection system is implemented. 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 efficiency.

During the experiment, the lower surface of the forearm gently rested an aluminum plate that contained an aperture to which a coverslip was glued. A drop of immersion oil was placed between the microscope objective and the cover slip. A drop of water was placed between the skin and the cover slip. Optical sections were acquired from difference depths by a piezo-motor interfaced to a computer which translated the microscope objective in vertical steps of 0.25 microns. Typical experiments were performed with 780 nm excitation light and an incident power of 10-15 mW.

6. Microscopy of human skin in vivo: tandem scanning confocal microscopy

Real-time optical sections of human skin were acquired from the lower forearm of the same volunteer, using tandem scanning confocal microscope which has been previously described [4-11]. Optical sectioning is due to spatial filtering in the sets of conjugate holes in the Nipkow disk of the tandem scanning confocal microscope.

The key feature of this microscope is a microscope objective which is fixed in position, and a annular ring which makes contact with the skin and moves under computer control along the z-axis. This device stabilizes the skin during image acquisition and displaces the skin with respect to the focal plane of the microscope. A 50X/0.85 NA Nikon oil immersion objective lens was used. A drop of microscope immersion oil (n=1.518) was placed between the skin surface and the tip of the microscope objective. The light source was a 250 W halogen lamp, which transmitted incoherent light (400-700 nm) via a fiber optic light guide to the microscope. The optical sections were acquired in 1 micron vertical steps. Real-time video frames were captured with a low-light-video camera (Dage MTI SIT68) coupled to a Sony

#27001 - $15.00 US Received October 20, 2000; Revised December 05, 2000

Hi8 video recorder. Rapid video recording of the Z-series through the lower aspect of the forearm avoided shifts caused by subject movement and blood flow pulsations. Two video frames were averaged, and the average was digitized, providing a stack of 64 optical sections in one micron vertical steps. The field of view of each image was 190 microns at the skin. The images were digitized in a format of 512 x 512 pixels (8 bits) and stored in the TIFF format.

7. Results

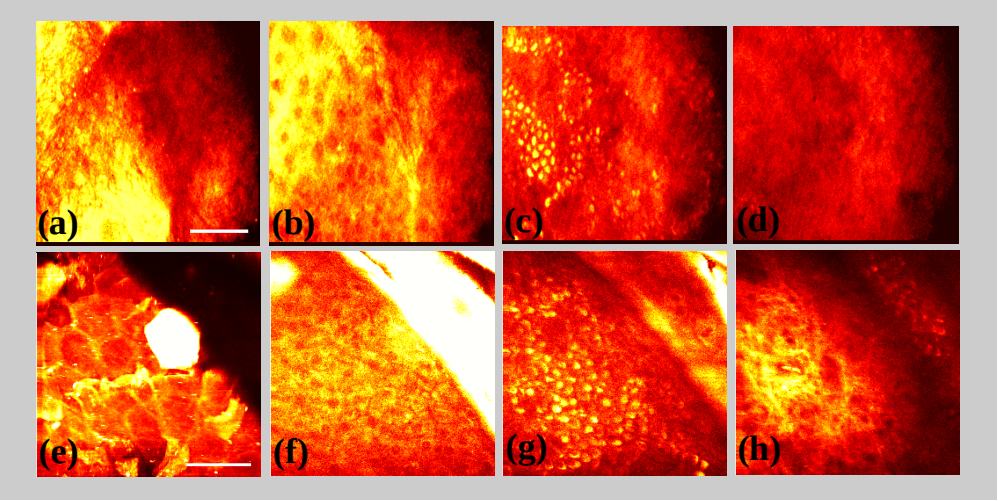

Fig. 3. Frontal sections of human skin in vivo acquired with reflected light confocal microscopy (a-d) and acquired from multi-photon excitation microscopy with excitation at 780 nm (e-h). Images were acquired from the volar surface of the forearm at similar positions with the two techniques described in the Methods section. Images were acquired at the regions: 10 microns below surface in stratum corneum (a and e), cells of stratum spinosum (b and f), cells of basal layer (c and g), and within the dermis (d and h). Scale bars show 50 microns.

In order to compare images acquired with reflected light confocal microscopy and multi-photon excitation microscopy we have used both techniques to image the lower surface of the human forearm of the same human volunteer in vivo. Similar but not identical fields were imaged. We will present a comparison of images acquired with both techniques.

Figure 3 shows frontal sections of human skin in vivo acquired with both reflected light confocal microscopy (upper row) and multi-photon excitation microscopy (bottom row). While there are similarities there are also significant differences between these sets of images. The images at the left of the figure are near the skin surface and those at the right of the figure were acquired from deeper regions of the skin.

The upper row stack is acquired with reflected light confocal microscopy. Image 3a shows an optical sections about 7 microns below the surface of the stratum corneum. It is very difficult to observe the individual keratinocytes in the stratum corneum. Image 3b shows the cells in the stratum spinosum. The cell nuclei are less reflective than the extranuclear regions and appear darker in reflected light confocal microscopy. Image 3c shows the individual basal cells in the basal layer. The basal cells are shown in high contrast due to the aggregates of melanin associated with the cells as previously reported [7]. Images acquired below the basal cell layer (image 3d) show weak contrast when illuminated with white light.

#27001 - $15.00 US
Received October 20, 2000; Revised December 05, 2000

The images on the lower row of Figure 3 were acquired with multi-photon excitation microscopy using coherent light at 780 nm. Optical sectioning is due to the physics of the multi-photon excitation process as implemented in the microscope. Contrast is provided from fluorescence of intrinsic fluorophores in the skin. In the epidermis, fluorescence is predominately from the reduced pyridine nucleotides, NAD(P)H. Since NAD(P)H is an intrinsic cellular cofactor for enzymatic reactions involving oxidative metabolism is serves as an intrinsic probe of cellular oxidative metabolism and therefore provides for functional imaging as well as morphological imaging [8, 28, 29]. In the dermis the contrast is provided by the fluorescence of the collagen and elastin.

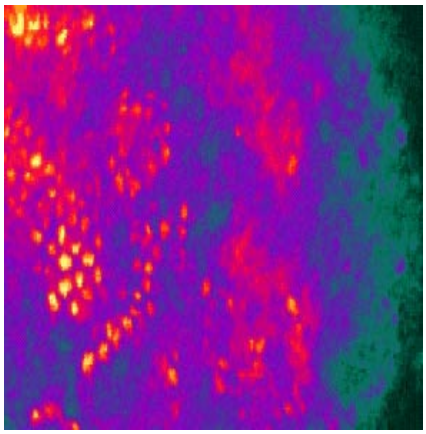

Fig. 4. (2.29 Mb) Movie of stacks of optical sections from human skin in vivo. Optical sections were acquired with reflected light confocal microscopy. Intensity is mapped into colors to enhance visualization, high intensities are yellow and red, low intensities are green and black. The horizontal length of this field is 175 μm.

Image 3e shows the keratinocytes in the stratum corneum at a level of about 10 microns below the surface. The borders of the individual squames are clearly shown in high contrast. Image 3f shows cells in the stratum spinosum. Image 3g shows a large field of basal epithelial cells. The basal cells are shown in high contrast due to the aggregates of melanin associated with the cells. Image 3h shows a significant difference between the two methods. Images acquired below the epidermal-dermal junction show markedly improved contrast with the multi-photon excitation microscope. In image 3h the fluorescence of the collagen and elastin fibers provides the high contrast in the image.

Figure 4 is a movie of a sequential set reflected light confocal microscopy of human skin in vivo. The first image is at the skin surface, and subsequent images are deeper towards the dermis. Figures 5 is a movie of multi-photon excitation fluorescence microscopy of a sequential set of images human skin in vivo. These two movies of stacks of optical sections of human skin provide an enhanced method to present highly complex three-dimensional data. Alternative methods to present three-dimensional views of human skin include: the use of color depth coding [8], red and green anaglyphs [9], and volume visualization [10]. Among the three techniques the use of movies of sequential optical sections is the simplest and corresponds to the serial acquisition of optical sections. The movies can be played in either direction, from the skin surface toward deeper regions or in reverse. Individual optical sections can be viewed by stopping the movie. An interesting effect is the spatial integration of the moving optical sections which provides the observer with a “three-dimensional” view of the human skin in vivo. Alternative technique to present three-dimensional images of human skin were previously selected because there was not an opportunity to publish movies

#27001 - $15.00 US Received October 20, 2000; Revised December 05, 2000

within the paper but this limitation has been circumvented by the availability of on-line publishing.

8. Discussion

This paper compares reflected light tandem scanning confocal microscopy with multi-photon excitation microscopy for the microscopic imaging of human skin in vivo. The structures in the human dermis observed by in vivo two-photon imaging is similar to that of excised mouse skin [27]. Comparison with the data obtained from the previous reflected light confocal microscopic in vivo study [9,10], shows that multi-photon excitation microscopy permits additional structures to be observed including the corneocytes in the stratum corenum and collagen/elastin fibers in the dermis.

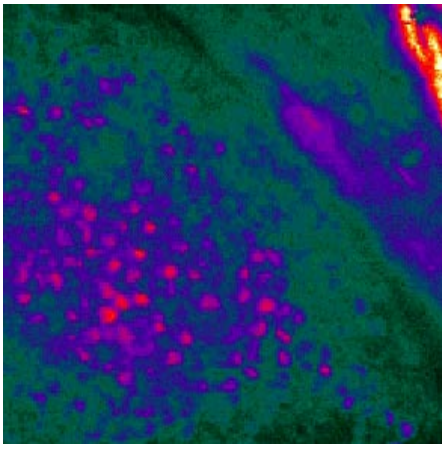

Fig. 5. (1.45 Mb) Movie of stacks of optical sections from human skin in vivo. Optical sections were acquired with multi-photon excitation microscopy with excitation at 780 nm. Intensity is mapped into colors to enhance visualization; high intensities are red and yellow, low intensities are dark red. Reconstruction shows the cells 10 microns below the surface within the stratum corneum, the cells of the basal layer, and structures within the dermis. The horizontal length of this field is 175 μm.

The tandem scanning reflected light confocal microscope acquired images at video rates. In principle the confocal microscope can image in both the fluorescence mode and the reflected light mode. The typical implementation of the multi-photon excitation microscope acquired images a significantly slower speeds that video rate and is limited to fluorescence imaging mode. These limitations have recently been overcome by new instrumental development [30]. Other groups have also developed a fast scanner to acquire multi-photon excitation images at video rate which is based on previous design [31,32].

Another important factor is photodamage which is not an issue in tandem scanning confocal microscopy where the average light flux is low because of the large illumination area. However, the thermal damage in a two-photon microscope resulting from one-photon absorption in the diffraction limited volume can be a significant factor because of the much higher light flux (1.5×10^5 W/cm^2). In general, when biochemical information is not required, reflected light confocal microscopy may be preferred for morphological studies of skin on the basis of cost, simplicity of instrumentation, and ease of use.

#27001 - $15.00 US Received October 20, 2000; Revised December 05, 2000

Acknowledgments

We thank G. Gonnord and P. Corcuff for assistance in acquisition of the reflected light confocal images of human skin in vivo. This work was supported by grants from ACS RPG9805801CCE and Unilever Corporation.

#27001 - $15.00 US Received October 20, 2000; Revised December 05, 2000