JOURNAL OF BIOMEDICAL OPTICS 2(4), 437–445 (OCTOBER 1997)

RAPID OBSERVATION OF UNFIXED, UNSTAINED HUMAN SKIN BIOPSY SPECIMENS WITH CONFOCAL MICROSCOPY AND VISUALIZATION

Barry R. Masters,[†] David J. Aziz,[‡] Arthur F. Gmitro,[*] James H. Kerr,[**] Terence C. O'Grady,[**] and Leon Goldman[**]

[†]Uniformed Services University of the Health Sciences, Department of Anatomy and Cell Biology, Bethesda, Maryland 20814; [‡]University of Arizona, Optical Sciences Center, Tucson, Arizona 85721; [*]University of Arizona, Optical Sciences Center, Department of Radiology, Tucson, Arizona 85721; [**]Naval Medical Center, Department of Dermatology, San Diego, California 92134

(Paper JBO-137 received Feb. 7, 1997; revised manuscript received June 30, 1997; accepted for publication July 21, 1997.)

ABSTRACT

The use of reflected light confocal microscopy is proposed to rapidly observe unfixed, unstained biopsy specimens of human skin. Reflected light laser scanning confocal microscopy was used to compare a freshly excised, unfixed, unstained biopsy specimen, and *in vivo* human skin. Optical sections from the *ex vivo* biopsy specimen of human skin and *in vivo* human skin were converted to red-green anaglyphs for three-dimensional visualization. Contrast was derived from intrinsic differences in the scattering properties of the organelles and cells within the tissue. Individual cellular layers were observed in both tissues from the surface to the papillary dermis. Confocal microscopy of an unfixed, unstained biopsy specimen showed cells and cell nuclei of the stratum spinosum. Confocal microscopy of *in vivo* human skin demonstrated optical sectioning through a hair shaft on the upper hand. The combination of reflected light confocal microscopy and three-dimensional visualization with red-green anaglyphs provides a rapid technique for observing fresh biopsies of human skin. © *1997 Society of Photo-Optical Instrumentation Engineers.* [S1083-3668(97)00904-0]

Keywords laser scanning confocal microscope; skin biopsy; three-dimensional confocal microscopy; reflected light skin imaging.

(*To view red and green anaglyphs in Figs. 4b and 6a–d, hold the supplied red and green glasses in front of the eyes. The green filter must be held over the right eye to obtain the correct depth visualization.*)

1 INTRODUCTION

The use of light microscopy to image *in vivo* human skin has been suggested as a potential noninvasive tool for clinical dermatology by Goldman.[1,2] Noninvasive optical techniques may offer an alternative to the standard technique of histopathology. They do not create a wound and do not result in scarring of the body. They also may offer the possibility for large area scanning of the body that is not possible with standard invasive methods. *In vivo* microscopic examination of pigmented tumors, cutaneous vascularization, and parasites (such as scabies mites) are proposed for optical observation. In order to validate the noninvasive optical technique, further studies and clinical trials are required to compare clinical observations from *in vivo* confocal microscopy and those from clinical biopsies.

Histological technique involves the following steps: (1) cutting the tissue to obtain a biopsy specimen, (2) fixing and staining the sample, and finally (3) microscopic observation. Typically, a biopsy specimen is microscopically observed as a series of two-dimensional slices with light microscopy.

The rapid development of confocal microscopes has permitted noninvasive optical sectioning of human skin and thereby advanced the field of noninvasive optical observation in dermatology. A confocal microscope forms thin optical sections that can be used for three-dimensional visualization of human skin. There are several general references for confocal microscopy.[3–6] Confocal light microscopy of human skin can work in either reflected light or a fluorescence light mode. Reflected light confocal microscopy forms contrast in images that depends on the differences in the refractive index of local regions of the object.

This paper proposes and illustrates the use of reflected light confocal microscopy to rapidly observe freshly excised, unfixed, unstained biopsy samples. The use of red-green anaglyphs to visualize the ex-

Address all correspondence to Barry Masters. E-mail: barrymas@bicwater.usuf1.usuhs.mil

1083-3668/97/$10.00 © 1997 SPIE

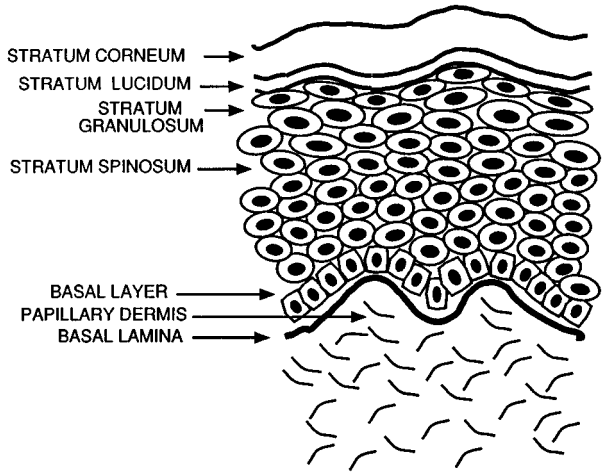


Fig. 1 Drawing of a vertical section of skin showing the following cell layers from the skin surface to the dermis: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, basal layer, and the papillary dermis. Individual cells forming the stratum corneum and the thin stratum lucidum are not shown in the drawing.

cised biopsy sample in three dimensions is demonstrated. Optical sections from a freshly excised biopsy sample and from *in vivo* human skin are compared and show similar features.

1.1 SKIN ANATOMY

The human skin can be divided into three layers: the epidermis, the dermis, and the subcutaneous tissue.[7] A drawing of a vertical section of human skin as seen in light microscopy is shown in Figure 1. For comparison, Figure 2 shows a vertical section of *ex vivo* human skin that had been fixed and stained according to standard methods of histopathology.

The innermost layer of the epidermis consists of a single layer of cuboidal cells called basal cells. These cells differentiate and migrate toward the surface of the skin when they eventually slough off. As they migrate to the skin surface, they become more and more stratified and finally form the cornified layer of the stratum corneum, which is composed of flattened and dead cells. The stratum corneum is highly reflective and about 10 to 20 μ thick, except for the skin on the feet and the palms, where it can be 500 to 600 μ thick.

The living epidermis contains five types of cells: keratinocytes, a few percent of dendritic cells, Langerhans cells, melanocytes, and rare Merkel cells. Keratinocytes are located in all cell layers. Melanocytes are located in the stratum basale. Langerhans cells are mostly located in the stratum spinosum (see Figure 1), and Merkel cells are found in or adjacent to the stratum basale.

1.2 CONFOCAL MICROSCOPY OF SKIN

Human skin presents specific problems for optical imaging. The skin surface is highly reflective and deeper layers contain a variety of cells as well as a vascular system. Skin thickness and color vary greatly among locations on the body and from individual to individual.

Goldman showed in 1951 that lesions that alter the surface of the skin can readily be observed with a low-power microscope.[1,2] The development of the confocal microscope has resulted in the ability to obtain images from deeper layers of human skin, and the development of computer techniques for three-dimensional visualization has helped to popularize the use and development of confocal microscopy for optical sectioning of living cells and tissues.

A tandem scanning confocal microscope was developed by Petran and co-workers to observe highly scattering tissues in real time.[8] Corcuff and co-workers have modified this tandem scanning microscope for the *in vivo* examination of human skin.[9–12] Recently, an *in vivo* confocal laser scanning microscope was developed and commercialized for video-rate imaging of *in vivo* human skin.[13]

2 METHODS

2.1 BIOLOGICAL SPECIMENS

An *ex vivo* human biopsy specimen was obtained with the approval and supervision of the Chair of the Department of Dermatology of the Naval Medical Center in San Diego, California. The specimen was freshly excised, from the upper back, unfixed, unstained, and immediately observed with a confocal microscope. The *in vivo* image was obtained by confocal microscopic imaging of the back of the hand of one of the authors, who was fully aware of any potential risks of the procedure.

2.2 CONFOCAL MICROSCOPY

A Zeiss laser scanning confocal microscope (LSM 10) with an air-cooled argon laser was used for these studies and was placed in a room adjacent to where the biopsy sample was obtained. We made the confocal observations within 1 min of excising the sample. In order to permit optical imaging at the deeper layers of the skin, we used combined argon-laser lines of 488 nm and 514 nm for image acquisition both *in vivo* and *ex vivo*. Images could be acquired to about 100 μ below the surface of the skin. Optical sections near the surface of the *ex vivo* biopsy sample appeared similar at excitations of 488 nm or at 514 nm. Chromatic aberrations did not noticeably affect image quality.

The confocal microscope was used in the reflected light mode for imaging the skin. Immersion oil was placed between the skin and the 63× (NA 1.4) oil immersion microscope objective used in these experiments. Each image of 512×512 pixels required 2 s to acquire. The pinhole was set at $p=20$, which resulted in a lateral resolution of about 0.58 μ. The laser power incident on the skin was 40 mW.

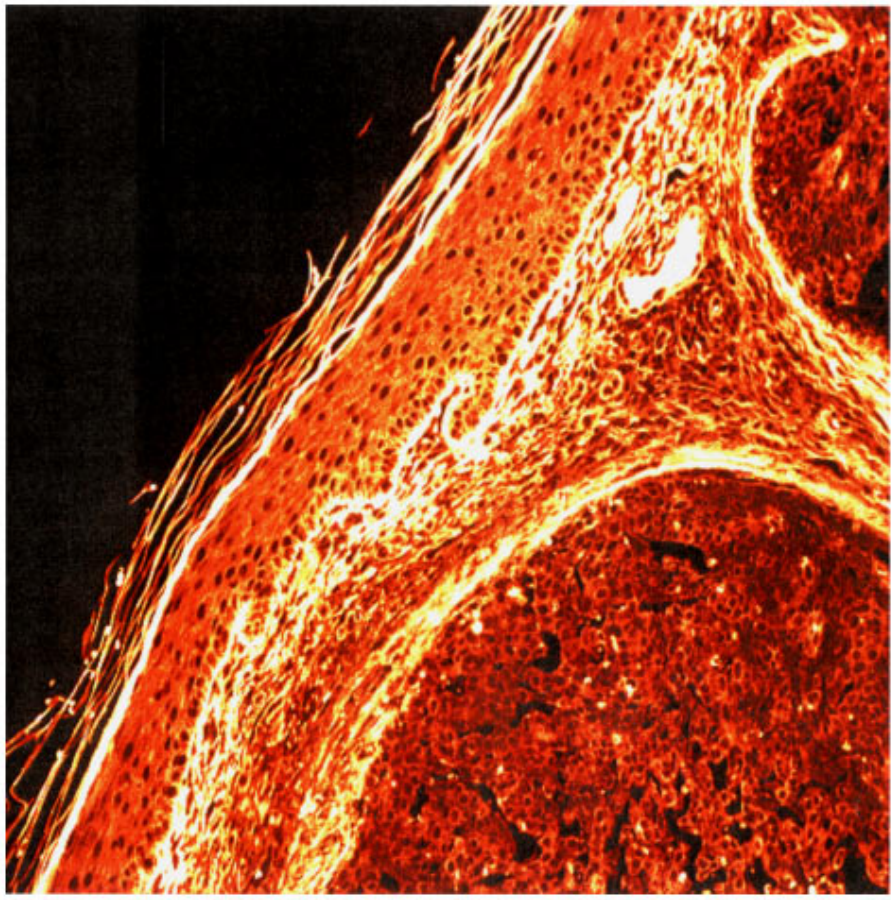
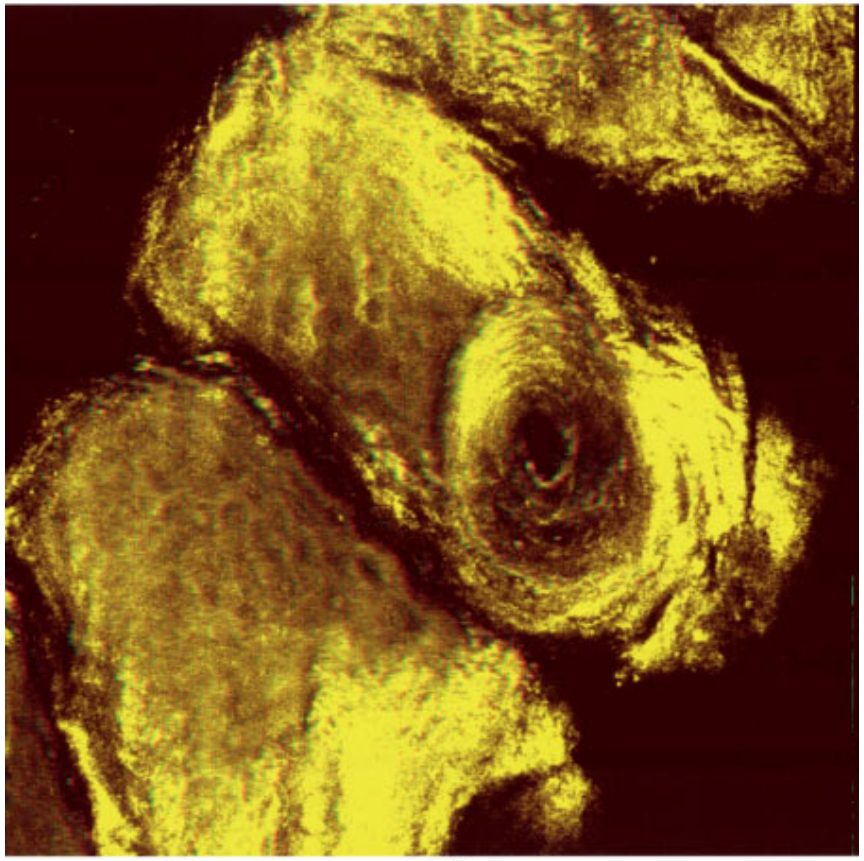


Fig. 2 Image of a fixed, stained vertical section of human skin. This is a classical biopsy specimen from the upper arm. The layers of the stratum corneum have become separated in the preparation of the section. Microscope objective 10×, 0.3 NA. Horizontal field width is 1390 μ.



(b)

Fig. 4 (Continued.)

COLOR PLATE

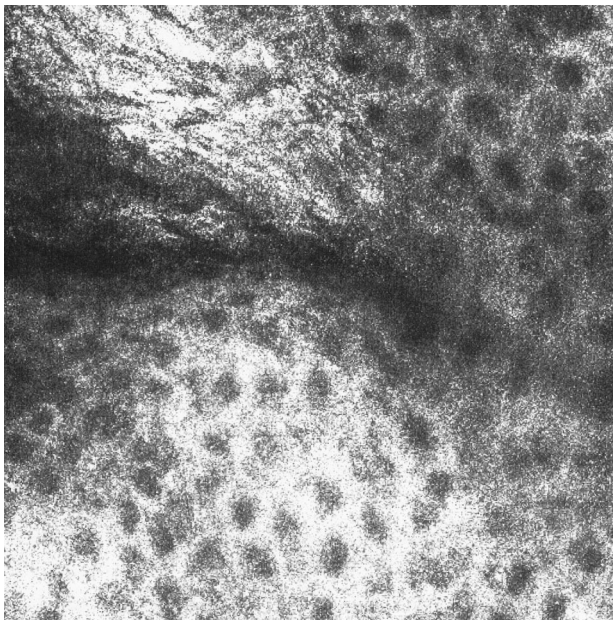
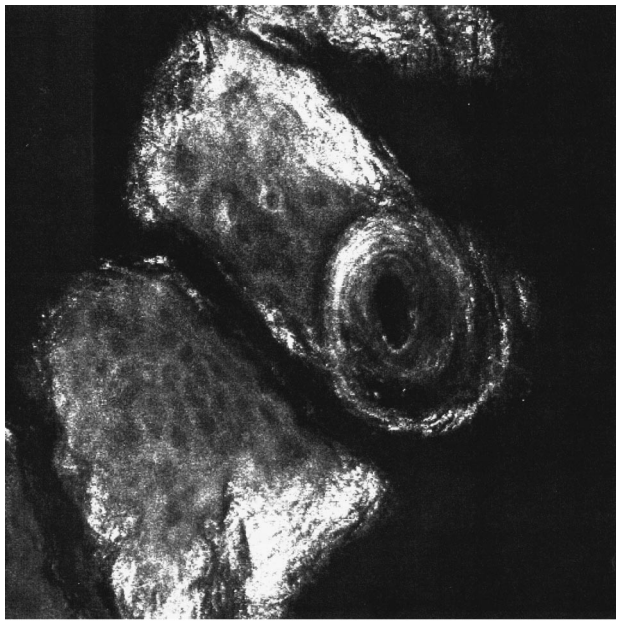


Fig. 3 Confocal reflected light image of *in vivo* human skin on the back of the hand. The bright cells with dark nuclei of the stratum spinosum are shown. On the upper left region of the image is the stratum lucidum. The depth of the optical section is about 100 μ. Microscope objective 63×, 1.4 NA, oil immersion. Horizontal field width is 225 μ.



(a)

Fig. 4 Confocal reflected light image of *in vivo* human skin on the back of the hand. (a) The image is an *en face* view of an optical section through a hair shaft with its concentric rings surrounding a central core. The dark nuclei of the stratum spinosum are shown. Depth of the optical section is about 50 μ. Microscope objective 63×, 1.4 NA, oil immersion. Horizontal field width is 225 μ. Fig. 4(b) A red-green anaglyph of a stack of optical sections of the same field as in Fig. 4(a) showing the three-dimensional visualization of the *in vivo* human skin.

After excision, the biopsy specimen was immediately placed on the stage of the microscope to acquire a series of optical sections from the surface of the tissue through the depth of the specimen. The specimen was oriented on the stage of the laser scanning confocal microscope so that the plane of the skin surface was parallel to the plane of the microscope stage. This permitted the acquisition of a stack of *en face* images, parallel to the surface of the skin, and extending from the skin surface to the papillary dermis. Image quality was enhanced by frame averaging four images for each optical section. This increased the signal-to-noise ratio of the final image. Frame averaging was used only for the freshly excised sample.

3 RESULTS

Both the biopsy sample and the *in vivo* skin were observed under the same experimental conditions. Figures 3 and 4 show images of *in vivo* human skin. Figure 3 is a typical optical *in vivo* section and shows cells with high reflectivity and dark nuclei. In the center of the image is a cleft, which appears dark due to low reflectivity. Figure 4(a) shows an optical section through a human hair shaft and the dark nuclei of the stratum spinosum. In 4(b) (color plate) the three-dimensional visualization of a series of five optical sections from the same field as Figure 4(a), but at different depths, is shown as a red and green anaglyph. *In order to view red and green anaglyphs, it is necessary to place a pair of red and green glasses in front of the eyes; the green filter must be placed over the right eye to obtain the correct depth visualization*.

Figures 5 and 6 show images obtained from the *ex vivo* specimen. Figures 5(a) through 5(c) show the specimen at increasing depths from the surface. Since the specimen was not completely flat, the optical section cut across cell layers in different regions of the specimen. Figure 5(d) shows the cells in the stratum spinosum, which is located just above the papillary dermis. The large cells of the stratum spinosum with their dark nuclei are easily observed. Figure 5(e) is an optical section across the papillary dermis and shows the rosettes of highly reflective basal epithelial cells.

The three-dimensional nature of the biopsy specimen is demonstrated with the use of red and green anaglyphs. Figures 6(a) through 6(c) (color plate) show the upper 50 μ of the specimen. The surface stratum corneum reflects much of the light, and the underlying cell layers are observed with difficulty. Figure 6(d) (color plate) is an interesting example of the three-dimensional visualization of the papillary dermis and shows the clusters of the basal epithelial cells. It was not possible with the anaglyph visualization method to show the full thickness of the biopsy specimen, therefore red and green anaglyphs were constructed from the surface layers and for the deeper layers. We did not succeed in imaging any cellular structures at depths deeper than the papil

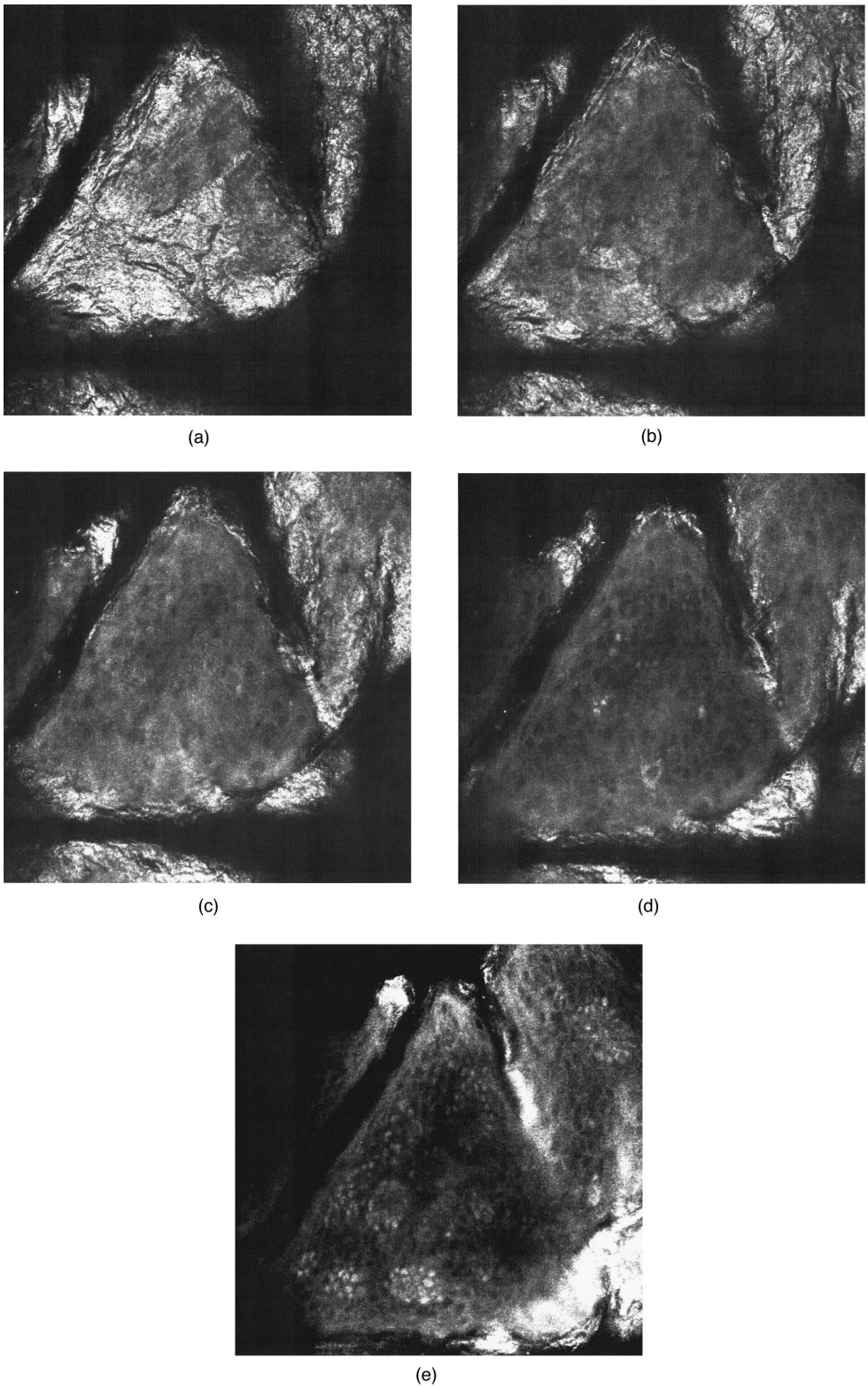
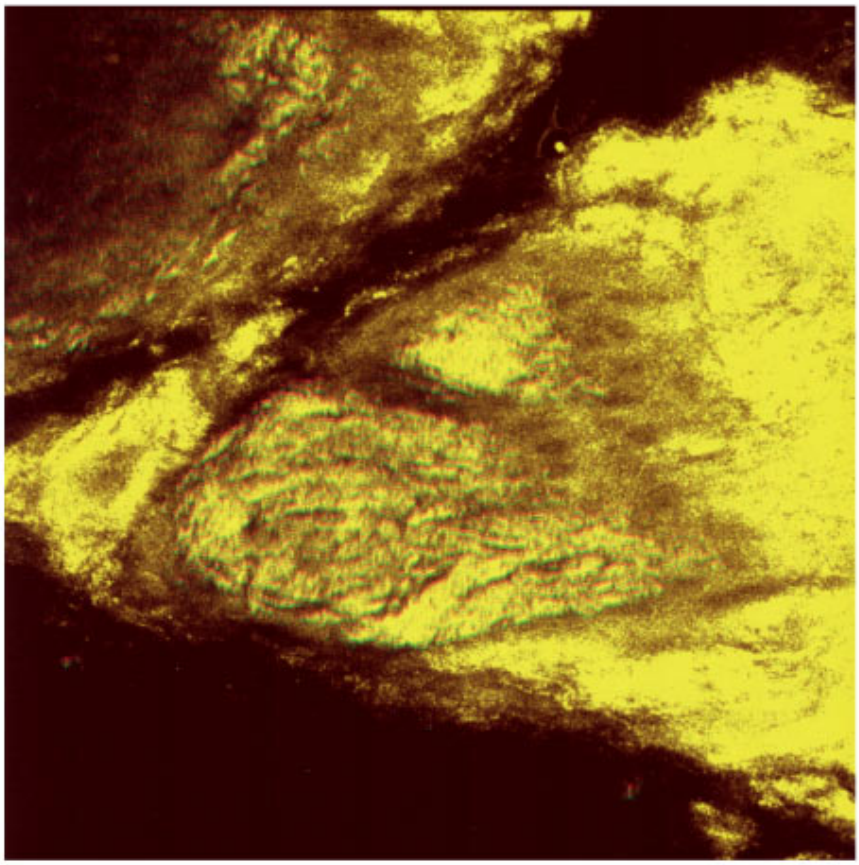


Fig. 5 A series of optical sections of a freshly excised, unfixed, unstained specimen of human skin from the upper back acquired with a confocal microscope. (a) An optical section showing the skin surface. The highly reflective stratum lucidum is shown. The optical section shows the cells just below the stratum lucidum. (b) Optical section shows a larger view of the cells just below the stratum lucidum. (c) An optical section that is slightly deeper than the previous one illustrating the optical sectioning capability of the microscope. (d) An optical section at a depth of about 85 μ showing the tops of the papillary dermis and the large cells of the stratum spinosum. (e) A deeper section showing the papillary dermis with the highly reflective basal epithelial cells. Microscope objective 63×, 1.4 NA, oil immersion. Horizontal field width is 225 μ.

Fig. 6 Red and green anaglyphs of stacks of optical sections of freshly excised, unfixed, unstained human skin acquired with a confocal microscope. Red and green colored glasses are required to view the anaglyphs. The green lens should cover the right eye. The anterior regions of the skin will appear closest to the observer. (a) Anaglyph showing deep ridges in the skin sample. The surface of the stratum corneum is closest to the observer. (b) Anterior regions of the skin, the middle and deeper regions that contain the stratum spinosum layer. (c) Regions that contain the stratum spinosum layer from a different location on the sample. (d) Three-dimensional visualization of the papillary dermis. The highly reflective basal epithelial cells are readily seen. Microscope objective 63×, 1.4 NA, oil immersion. Horizontal field width is 225 μ.

COLOR PLATE

(d)



(c)

Fig. 6 (Continued.)

COLOR PLATE

lary dermis for either the *ex vivo* biopsy specimen or the *in vivo* confocal microscopy.

4 DISCUSSION

4.1 ADVANTAGES

The standard technique for obtaining a biopsy specimen of human skin involves cutting the skin and then fixing, staining, and sectioning the tissue to form vertical sections. While it provides a vertical section of the full thickness of the tissue, this technique is invasive and leaves a scar.

An advantage of an optical biopsy technique is that it is noninvasive, which is a potentially important advance for a number of reasons. An optical biopsy could be used to screen a wide area of the body and since it does not leave scars, could be used with greater frequency for general dermatological screening. This would require the development of a large area scanning instrument. A further advantage is that *en face* optical sections can be acquired which can then be used with an appropriate computer technique to generate a three-dimensional visualization of the skin volume, which would provide the clinician with more information than a few vertical sections.

4.2 DISADVANTAGES

There are two aspects to the limitations of optical biopsy techniques: the development of instrumentation for optical biopsy of *in vivo* human skin and the development of visualization techniques for the presentation of the images. Since the quality of the image acquisition strongly determines the quality of the three-dimensional visualization, it is pertinent to cite some of the difficulties with noninvasive optical imaging of human skin.

The optical instrument should be able to acquire an image in a short enough time so that motion of the skin and underlying tissues does not degrade the image sharpness. Unless specific regions of the skin can be held stationary, such as the forearm, or the back of the hand, as imaged in the present study, a system that can acquire images at a video rate is desirable.

The safety of the diagnostic procedure is of primary concern. Therefore the power delivered to the skin must be limited to a level that does not cause physical or biological damage. In the present study, the power at the skin surface was 40 mW. We did not observe any morphological changes in the skin following several minutes of repeated observation of the same volume of *in vivo* human skin. This does not rule out biochemical changes or cellular damage that was not detected following several minutes of exposure to the light from the laser scanning confocal microscope. There could be long-term changes in the skin that were not observed following our microscopy.

The depth of penetration of the optical instrument is an important consideration. Factors that affect the depth of penetration include the power of the light incident on the skin, the wavelength of the incident light, and the amount of pigmentation in the skin. Penetration is greater for less pigmented skin. Higher power and longer wavelengths (only into the near IR) of light may result in greater penetration of the tissue. The use of longer wavelengths of light permit deeper layers of human skin to be observed *in vivo*.[13] Figure 5(e) shows an optical section obtained at the level of the papillary dermis. Deeper optical sections did not show sufficient contrast to allow identification of cellular structures at depths below the papillary dermis. For comparison, the image in Figure 2 of a vertical section that was excised, fixed, and stained for light microscopy provides a high-contrast vertical section of the full thickness of the skin in the specimen.

5 CONCLUSION

The study described in this paper used a laser scanning confocal microscope to rapidly observe a freshly excised, unfixed, unstained biopsy sample from human skin. We also demonstrated the use of red-green anaglyphs to visualize human skin. It is proposed that the combination of these techniques could provide rapid observation of fresh biopsies of human skin.

Acknowledgment

This work was supported by National Institutes of Health Grant EY-06958 (BRM).

REFERENCES

1. L. Goldman and W. Younker, ''Studies in microscopy of surface of skin. Preliminary report of techniques,'' *J. Invest. Dermatol.* **9**, 11–16 (1947).
2. L. Goldman, ''Some investigative studies of pigmented nevi with cutaneous microscopy,'' *J. Invest. Dermatol.* **16**, 407–427 (1951).
3. B. R. Masters, ''Confocal microscopy: history, principles, instruments, and some applications to the living eye,'' *Comments Mol. Cell. Biophys.* **8**, 243–271 (1995).
4. B. R. Masters, ''Selected papers on confocal microscopy,'' The International Society for Optical Engineering, Bellingham, WA (1996).
5. T. Wilson, *Confocal Microscopy*, T. Wilson, Ed., Academic Press, London (1990).
6. R. H. Webb, ''Confocal optical microscopy,'' *Rep. Prog. Phys.* **59**, 427–471 (1996).
7. K. S. Stenn, ''The skin,'' in *Cell and Tissue Biology*, L. Weiss, Ed., pp. 541–572, Urban and Shwarzenberg, Baltimore, MD (1988).
8. M. Petran, M. Hadravsky, M. D. Egger, and R. Galambos, ''Tandem scanning reflected light microscope,'' *J. Opt. Soc. Am.* **58**, 661–664 (1968).
9. C. Bertrand and P. Corcuff, ''*In vivo* spatio-temporal visualization of the human skin by real-time confocal microscopy,'' *Scanning* **16**, 150–154 (1994).
10. P. Corcuff, G. Gonnord, G. E. Pierard, and J. L. Lévêque, ''*In vivo* confocal microscopy of human skin: a new design for cosmetology and dermatology,'' *Scanning* **18**(3), 351–355 (1996).
11. P. Corcuff, C. Bertrand, and J.-L. Lévêque, ''Morphology of

human epidermis *in vivo* by real-time confocal microscopy,'' *Arch. Dermatol. Res.* **285**, 475–481 (1993).
12. P. Corcuff and J.-L. Lévêque, ''*In vivo* vision of the human skin with the tandem scanning microscope,'' *Dermatology* **186**, 50–54 (1993).
13. M. Rajadhyaksha, M. Grossman, D. Esterowitz, R. H. Webb, and R. Rox Anderson, ''*In vivo* confocal scanning laser microscopy of human skin: melanin provides strong contrast,'' *J. Invest. Dermatol.* **104**, 946–952 (1995).