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Skin Imaging With Reflectance Confocal Microscopy

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Confocal microscopy is a new imaging modality for noninvasive real-time tissue imaging with high resolution and contrast comparable with conventional histology. Application of this technology to skin imaging during the last decade has been an exciting advance in dermatology, allowing a virtual widow into living skin without the need for a conventional biopsy or histologic processing of tissue. High-resolution noninvasive skin imaging with confocal microscopy has potential broad applications in the clinical and research arenas, including differentiating between benign and malignant skin lesions, tumor margin mapping, monitoring response to medical or surgical treatments, and pathophysiologic study of inflammatory processes.
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The application of confocal microscopy for optical sectioning of solid tissue noninvasively developed out of a desire to visualize cells in a dynamic way. The conventional manner of examining tissue requires tissue fixation, sectioning, and staining before microscopic observation of the static tissue and this processing may introduce artifacts such as tissue shrinkage. In addition, classical serial sectioning of tissue leads to destruction of the sample whereas the optical sectioning property of the confocal microscope preserves tissue integrity. Furthermore, the optical sections of skin are acquired without trauma or scar formation from a biopsy. Because the imaging is in real time, different depths in the skin are imaged without the delay of conventional deeper histology levels. Finally, because the image is virtual, dynamic viewing and repeated imaging may be performed without alteration of the native skin. Other technologies are also being explored for skin imaging, such as optical coherence tomography,[1] high-frequency ultrasound,[2] and magnetic resonance imaging,[3] but confocal microscopy is the first to offer resolution at the single cell level.

Historical Development

Marvin Minksy described the first confocal microscope in 1957.[4] A tandem scanning confocal microscope was designed by Petran[5] in 1968 to optically section tissue in real time. The initial experiments with confocal microscopy were performed in vitro using a bright mercury lamp light source, and the technology was not applied to in vivo tissue imaging until the 1980s with advances in light sources, scanning, and computer technologies. Several research groups demonstrated the use of confocal microscopy for imaging live animal and human tissue in vivo showing detailed cellular images of teeth, cornea, kidney, liver, thyroid, adrenal gland, muscle, and connective tissue.[6-9] Research on in vivo imaging of human skin ultrastructure with confocal microscopy began in the early 1990s.[10-12] In 1995, Rajadhyaksha and colleagues[13] constructed a laboratory prototype video-rate confocal laser scanning microscope for rapid in vivo imaging of human skin. The use of a laser light source provided high illumination power and deeper penetrating wavelengths of near infrared to improve imaging capabilities. They reported the ability to image high-resolution nuclear and cellular level detail in normal human skin in vivo with good correlation to conventional histology. In 1999, Rajadhyaksha and coworkers[14] reported improved resolution, contrast, depth of imaging, and field of view with further advances in confocal instrumentation.

Principles of Confocal Microscopy

The basic premise of confocal microcopy is the selective collection of light from in-focus planes in the tissue. With the use of a low power near-infrared laser, a beam of light is focused tightly on a specific point in the skin. Light scattered or reflected from this illuminated point is collected through a

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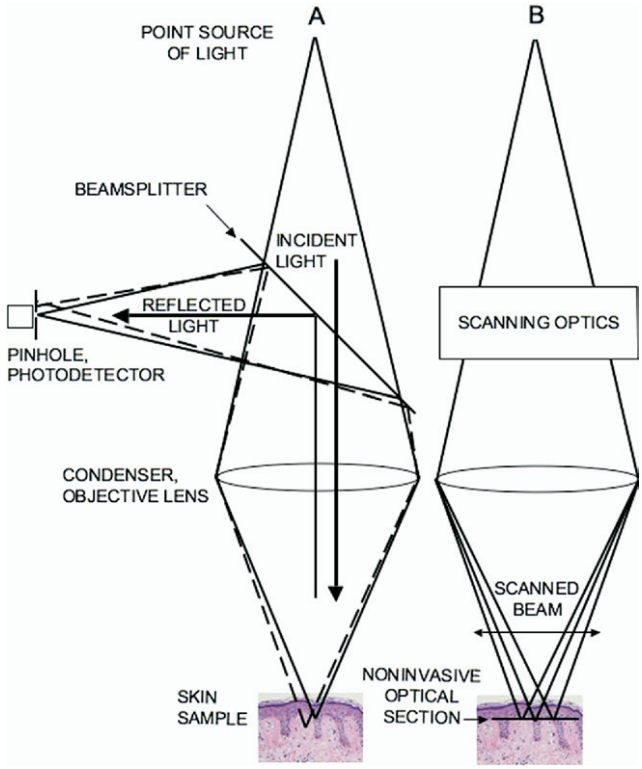

Figure 1 Basic principle of confocal microscopy. (A) A point source of light is mapped onto the tissue, and a point detection collects light only from the illuminated point (solid lines). Light from other points (dashed lines) is rejected by the pinhole aperture. In this manner, the confocal measurement is sensitive to only a small volume within a thick specimen. (B) Illustration of scanning, whereby the measured point is translated in a plane parallel to the surface of the sample. Sequential sampling leads to measurements that become pixels in an image. (Color version of figure is available online.)

pinhole sized aperture by a detector. The light source, illuminated point, and detector aperture are in optically conjugate focal planes (ie, confocal planes). This configuration (Fig. 1) allows for collection of light from the single in-focus plane and rejection of light from all out-of-focus planes. Factors affecting the depth of penetration of light include the wavelength of illumination, power of illumination, reflectivity of the superficial layers, and scattering properties of the dermis. Longer wavelengths will penetrate deeper because of decreased light scatter, although the resolution decreases as the wavelength increases.

The point source beam is scanned over a 2-dimensional grid to obtain an image of a thin plane or a thin optical section within the tissue. The optical section is oriented parallel (ie, en face) to the skin surface. The optical sectioning in confocal microscopy contrasts with the conventional orthogonal sections of histopathology that are oriented perpendicular to the skin surface. By translating the lens orthogonally relative to the skin, the confocal microscope images a series of en-face planes that are stacked vertically in depth with a very thin section thickness and high lateral resolution.

Optical reflectance imaging relies on the natural variations in refractive indices of tissue microstructures for contrast. The role of melanin as a contrast-forming agent in skin was reported by Rajadhyaksha and colleagues in 1995.[13] With the use of a laser scanning confocal microscope, normal human skin was imaged in vivo in different subjects. Individuals with darker skin and greater melanin content had a brighter appearance of keratinocytes, especially along the basal layer. In human skin, melanin is the strongest endogenous contrast source for confocal imaging. Other sources include keratin, mitochondria and cytoplasmic organelles, chromatin in the nuclei, and collagen in the dermis.

Confocal Microscopy Instrumentation

The original confocal microscope demonstrated feasibility but, as with any new technology, was a large and immobile bench top instrument with suboptimal skin stabilization and lens-to-tissue interface that made imaging human skin relatively difficult.[15] A partnership between Lucid Inc (Rochester, NY) and Massachusetts General Hospital led to the development of a smaller portable unit with an improved microscope-to-human skin interface. The present commercially available confocal laser microscope uses a deeper penetrating near-infrared wavelength of 830 nm (diode laser) with an illumination power of 1 to 5 mW, which is safe on the tissue and causes no eye injury (Fig. 2). Water immersion objective lenses of magnifications 20X to 100X and numerical aperture (NA) 0.7 to 1.2 provide fields-of-view of 1 to 0.2 mm, lateral resolution of 0.5 to 1.0 μm and optical section thickness of 2 to 5 μm with illumination wavelengths of 800 to 1064 nm. A 30X objective lens of NA 0.9 that is routinely used provides a field of view of 0.5 mm, lateral resolution of 0.7 μm, and optical section thickness of 3 μm,[13,14] which is comparable with that of conventional histology sections. With this system, depth of imaging of normal skin approaches 250 to 300 μm, with good visualization of the epidermis, papillary dermis, and upper reticular dermis.

To enable visualization of larger areas of tissue with varying magnification, similar to that in histopathology, a 2-dimensional sequence of images are captured and software-stitched into a mosaic. Up to 16 $\times$ 16 images may be captured to create a mosaic that displays up to 8 $\times$ 8 mm of tissue, which corresponds to approximately 2X magnification. Zooming into the mosaics produces submosaics with higher magnifications, similar to the use of objectives with different magnifications when examining histopathology sections.

A water immersion objective lens is used because the refractive index of water (1.33) closely matches the 1.34 refractive index of the living epidermis.[16] This close approximation minimizes spherical aberrations and reduces loss of resolution and contrast when imaging deep in the skin. Typically water based gels are used as the immersion media reducing irregularities in refraction. The microscope has a mechanical fixture consisting of a metal ring to allow stable skin contact and imaging at varied topographic sites on the body. As the objective lens is moved orthogonally relative to the skin,

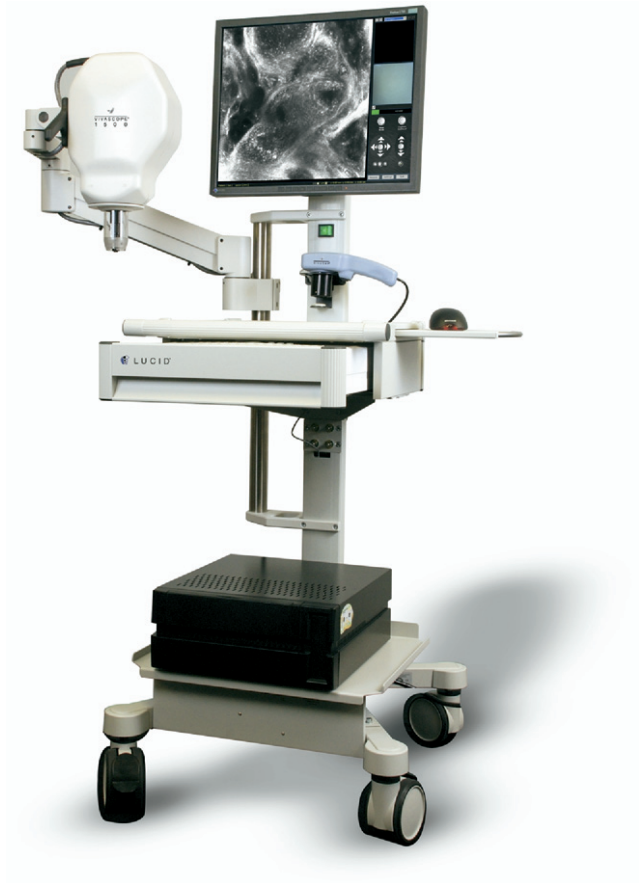

Figure 2 Confocal instrument: The VivaScope® 1500 is mounted on a cart with the VivaCam™ which produces dermatoscope-like wide field-of-view images. (Courtesty of Lucid, Inc. Rochester, NY). (Color version of figure is available online.)

different en-face levels of skin are imaged, analogous to tissue blocks in conventional histology. Live images may be captured either as single frames or as sequential frames to form videos to demonstrate dynamic events.

Confocal Imaging of Normal Skin

Confocal imaging of human skin in vivo[13,14,17] has shown good correlation with conventional histology. When imaging skin in real time, the most superficial layer of the epidermis, the stratum corneum, is first visualized. The stratum corneum appears as a brightly refractive image due to back scattering of light at the immersion medium-tissue junction. The granular layer appears next consisting of cells with bright grainy cytoplasm surrounding a large dark oval nucleus. The spinous layer presents as a tight honeycombed pattern of smaller cells. The deepest layer of the epidermis, the basal layer, is seen as bright clusters of cells at the dermal-epidermal junction. Below this junction, capillary loops can be visualized in the dermal papillae. Deeper imaging of skin reveals the papillary dermis with reticulated fibers and small blood vessels (Fig. 3). The superficial reticular dermis reveals matrix fibers, hair follicles, and sweat ducts. Huzaira and colleagues[17] studied regional variations in human skin with confocal microscopy in patients with varied skin phototypes and demonstrated ability to image differences in skin ultrastructure.

Confocal Imaging of Benign and Malignant Skin Lesions

Real-time confocal microscopy introduces the possibility of studying multiple scenarios noninvasively such as pathophysiology of inflammatory conditions and response to treatments. The major features of psoriatic skin, allergic skin reactions, and discoid lupus erythematosus[18-20] have been demonstrated with in vivo confocal microscopy. Aghassi and coworkers[21,22] reported use of in vivo confocal microscopy to noninvasively and sequentially evaluate dynamic changes after pulsed dye laser treatment of sebaceous hyperplasia and cherry angioma lesions.

The major microscopic features of several premalignant and malignant lesions with in vivo confocal microscopy also have been reported. The pathologic features of an actinic keratosis, including pleomorphism and architectural disarray, could be visualized with in vivo confocal microscopy and differentiated from adjacent normal skin.[23,24] The microscopic features of basal cell carcinomas have been correlated to conventional histology and follow up studies have shown high sensitivity and specificity for detecting this skin cancer with in vivo confocal imaging.[25,26] Goldgeier and coworkers[27] demonstrated the feasibility of monitoring the response of a basal cell carcinoma treated with imiquimod with confocal microscopy.

Analysis of a series of pigmented skin lesions has revealed distinct in vivo morphologic differences between benign and malignant lesions.[28-32] The melanin in pigmented skin lesions has a high refractive index, producing strong back-scatter and is therefore a natural source of contrast for confocal microscopy. Several authors were able to diagnose melanoma lesions with high sensitivity and specificity using confocal microscopy based on morphologic features. Characterization of confocal microscopy features of melanomas and nevi appears to improve diagnostic accuracy for melanocytic lesions that are difficult to diagnose.[32]

Confocal Imaging For Tumor Margin Mapping

The use of confocal microscopy to examine freshly excised skin specimens ex vivo with noninvasive optical sections was reported by Rajadhyaksha and colleagues[33] in 2001. Discarded Mohs surgery excision specimens with residual basal and squamous cell carcinoma tumors were washed in an exogenous agent, acetic acid, to enhance contrast and detection of the nuclei. Acetic acid causes compaction of chromatin within nuclei and makes the nuclei appear bright as the result of significant back-scatter in contrast to the dark nuclei in standard confocal imaging. The acetowhitened tissue was imaged with confocal microscopy and compared with con-

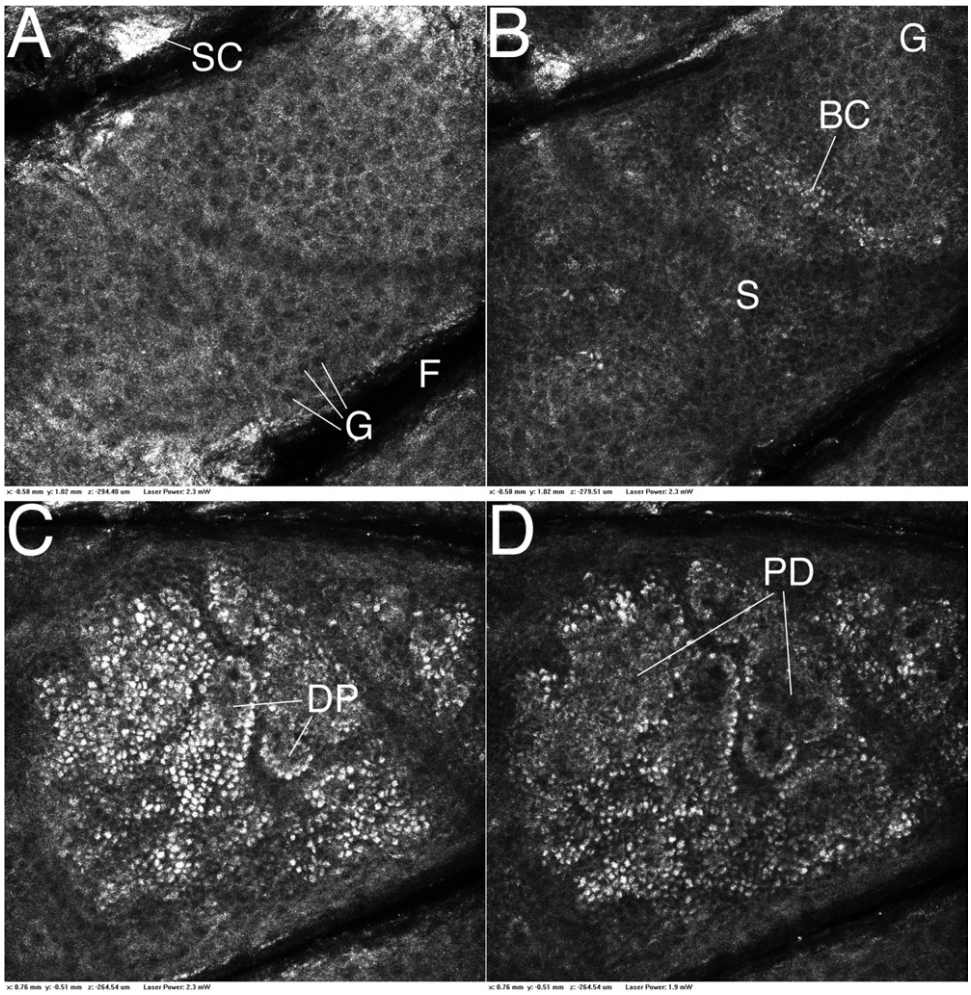

Figure 3 Normal skin in vivo. In A, the superficial layer includes granular cells (G) with dark nuclei, skin folds (F) and the bright stratum corneum (SC). In B, a deeper layer shows spinous cells (S) and pigmented basal keratinocytes (BC). C: the dermal papillae (DP) are visible at the next depth. D: the papillary dermis (PD) is visible beneath the dermal papillae. (Photographs from Christi-Alessi-Fox, Lucid, Inc).

ventional histologic sectioning. Acetowhitening strongly enhanced brightness and contrast of nuclei in basal cell carcinoma tumor nests relative to the surrounding dermis facilitating tumor detection. To examine the entire excision specimen, a sequence of images of adjacent fields was digitally captured. The images were stitched together using software to create a mosaic that showed the entire excision specimen equivalent to a 2X view. The mosaic is essentially a low-resolution map showing the overall location and morphology of the tumors and the high resolution images are used to inspect cellular detail.

In a large series of Mohs surgery excision specimens (of basal and squamous cell carcinomas) that were acetowhitened and imaged with ex vivo confocal microscopy, Chung and coworkers[34] demonstrated that confocal images correlated well with standard Mohs frozen section histology in terms of tumor location and shape and margins between normal and tumor tissue. The advantage of the confocal microscope was the ability to change the depth of imaging and follow skin structures dynamically rather than waiting for deeper levels in conventional histology. Nodular basal cell carcinomas were easily and consistently detected with confocal microscopy. Micronodular and infiltrative basal cell carcinomas were more difficult to visualize consistently because of smaller tumor aggregates and location deeper in the skin where the reflectance properties of the collagen compete with the acetowhitened nuclei. These results suggested that confocal imaging could potentially expedite detection of residual tumor in Mohs skin excisions. However, these early imaging methods did not yield consistent results.

Patel and coworkers[35] described advances in confocal instrumentation that further enhanced and refined ex vivo confocal imaging of excised surgical specimens. Because fresh excised tissue has complex shapes, it is difficult to mount for confocal microscopy. To overcome inconsistent confocal imaging that was experienced in the initial studies, a mechanical tissue fixture was designed to hold the specimen and precisely control flattening, tilt, sag, and stability of the tissue. Furthermore, improved algorithms and software were developed that produced seamless confocal mosaics simulating a 2X light microscopy overview of the Mohs specimen. Acetic acid concentrations and soaking times were also tested to

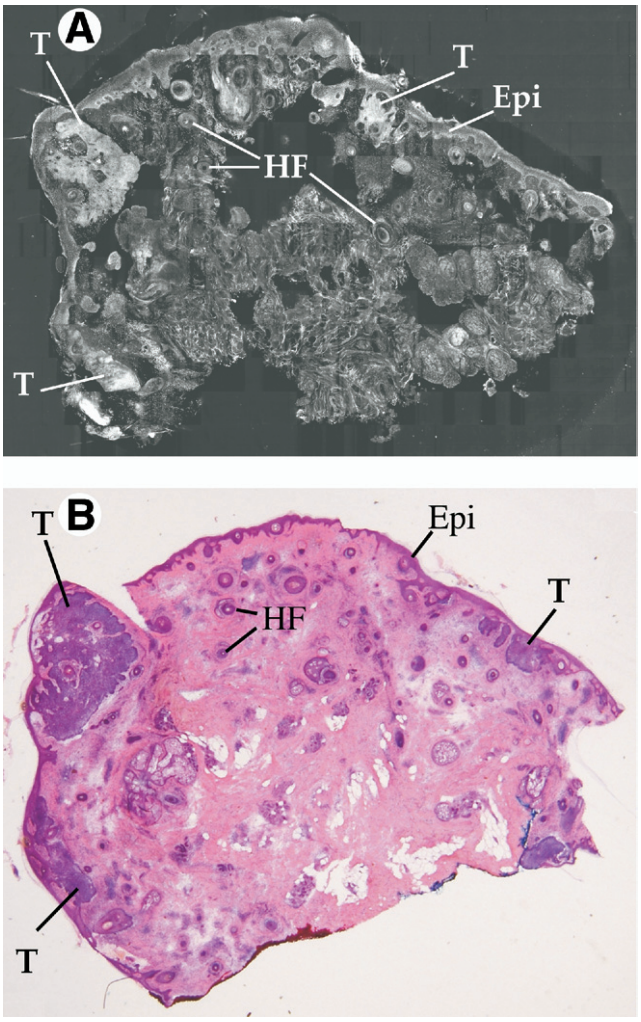

Figure 4 Confocal mosaic. Individual images are stitched together to create a confocal mosaic (A), which is roughly equivalent to 2X histology. Epidermis (Epi), hair follicles (HF), and basal cell carcinoma tumors (T) are visible, which correspond to conventional Mohs frozen sections (B) stained with hematoxylin and eosin. (Color version of figure is available online.)

determine optimal acetowhitening conditions. The tissue fixturing and mosaicing software developments led to significant improvements in the consistency of imaging and repeatability of mosaic quality (Fig. 4).

Gerger and coworkers[36,37] showed high diagnostic accuracy for basal cell and squamous cell carcinomas compared with normal skin with ex vivo confocal microscopy of excised Mohs specimens validating this imaging modality. Tannous and coworkers[38] studied the feasibility of in vivo confocal imaging during Mohs surgery for biopsy proven basal cell carcinomas. After application of aluminum chloride for hemostasis, the Mohs wound was imaged through a sterile ring. The authors reported aluminum chloride provided good contrast between tumor cells and the surrounding tissue facilitating tumor detection. Further study will be necessary to determine if confocal imaging within a live open wound can produce consistent images and high diagnostic accuracy in detecting tumors. Beyond Mohs surgery for skin cancers, confocal microscopy may enable rapid surgical pathology at the bedside in many settings. Potential examples include excisions of thyroid nodules, parathyroid glands and bone during oral and head and neck surgery, needle core-biopsies and lumpectomies of breast, and biopsies of liver, bladder and other tissues.[39-41]

Future Direction

The present limitations of reflectance confocal microscopy include the depth of imaging that is limited to the superficial dermis due to tissue-induced scattering and aberrations. The grayscale (black/white) contrast also lacks specificity for organelles and ultrastructure. Furthermore, the instrumentation is relatively expensive, complex, large, and difficult to use on awkward anatomical sites. However, laboratory research on understanding light scattering and aberrations within tissue and methods to correct for these effects continue to progress. Over time, advances may enable deeper imaging in skin. Meanwhile, the current commercially available technology is already advancing toward handheld confocal microscopes that are less expensive, more robust and easier to use on humans. Recently, novel confocal line-scanning microscopes demonstrated imaging of nuclear and cellular morphology in human epidermis.[42,43] Presently, the line-scanning microscopes are laboratory prototypes but they are much simpler than current point-scanning technology and may lead to a new class of handheld low-cost confocal microscopes for skin imaging.

Such advances will not, however, be limited to reflectance confocal microscopy. Similar to the use of multiple stains in conventional histology, multiple optical stains or modes of contrast may be necessary for skin imaging. Several other modes of contrast are being developed such as 1-photon and 2-photon fluorescence, autofluorescence, Raman scattering and Terahertz.[44-49] Such modes may be synergistically combined and hold promise for multimodal imaging of optical sections in skin with the ability to provide structure-specific contrast.[50,51] Multimodal confocal microscopy thus may offer enhanced diagnostic potential and application.

Summary

Real-time confocal microscopy offers the unique advantage of viewing in vivo cellular details in human skin noninvasively, safely, and rapidly at a resolution comparable to that of conventional histology. These features of confocal microscopy provide a powerful tool in the diagnosis and management of skin lesions and diseases. Multiple studies have already demonstrated excellent correlation between confocal microscopy and conventional histology for benign and malignant lesions. Further advances in confocal instrumentation, understanding mechanism of contrast in vivo and ex vivo, and continued correlation of confocal images with conventional histology will drive progress in confocal imaging and present new opportunities for clinical and research applications.

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