In vivo confocal scanning laser microscopy: comparison of the reflectance and fluorescence mode by imaging human skin

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Abstract. Optical, noninvasive methods have become efficient *in vivo* tools in dermatological diagnosis and research. From these promising imaging techniques, only the confocal scanning laser microscopy (CSLM) provides visualization of subsurface skin structures with resolutions similar to those of light microscopy. Skin annexes, as well as cutaneous cells from different epidermal layers, can be distinguished excellently. Currently, two forms of application have been established in dermatological practice: the reflectance mode, predominantly in the clinical field, and the fluorescence mode in dermatological research. Differences in both methods exist in the preparative protocol, in maximum imaging depth and, particularly, in the gain of contrast extraction. The reflectance mode demonstrates naturally occurring tissue components, whereas the fluorescent CSLM achieves contrast by administering fluorescence dye, representing the dynamic distribution pattern of the dye's fluorescent emission. Therefore, the reflectance and fluorescent modes highlight various skin microstructures, providing dissimilar *in vivo* confocal images of the skin. This permits different predications and information on the state of the tissue. We report the advantages and disadvantages of both optical imaging modes. The comparison was drawn by scanning human skin *in vivo*. Representative images in varying depths were obtained and analyzed; preparation procedures are shown and discussed. [DOI: 10.1117/1.2337294]

Keywords: lasers in medicine; confocal microscopy; noninvasive imaging; fluorescence imaging; reflectance imaging; skin morphology.

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1 Introduction

During the last years, confocal microscopy has brought substantial improvements in the imaging of biological tissue *in vivo*.[1] In dermatology, this noninvasive technique is used to perform nondestructive optical sectioning of human skin in progressive depth. This ability provides online visualization of surface and subsurface detail in living skin. Therefore, many dermatological studies have been performed using this promising scanning instrument: morphometric explorations,[2] skin penetration analysis,[3] and furthermore, diagnostical investigations for a number of clinically relevant skin disorders[4] were carried out. Altogether, they expose a vast field of application for CSLM in dermatological research and practice.

The first confocal scanning microscope was invited by Marvin Minsky for studying neutral networks in living brain.[5,6] Since then, there have been considerable developments and improvements in the light source and the computerization technology.[7,8] Briefly, today the combination of at least a laser light source, an optical system, and a spectrometer enables modern CSLM measurements to be performed. The confocal microscopic machinery focuses a laser light in a small point within the dermal tissue. Returning light from the focal plane is collected simultaneously and exploited to create an optical image. The system is designed to detect the light directly from the focal point and to prevent any scattered and reflected light from out-of-focus planes, enabling virtual sections of a thin horizontal tissue plane. For depth scan, the plane can be adjusted and positioned below the skin surface.[9]

The received high-resolution images demonstrate the histological architecture of the epidermis, as well as of the upper parts of the underlying dermis. The different epithelial cells (keratinocytes) can be remarkably distinguished by their typical size and shape; also their transition in morphology from skin surface to the papillary dermis can be observed. The various cutaneous layers can be reliably identified by their characteristic array and depth localization within tissue. *In vivo* confocal imaging offers real-time scan sequences with images obtained in horizontal view (*en face*) to the skin sur-

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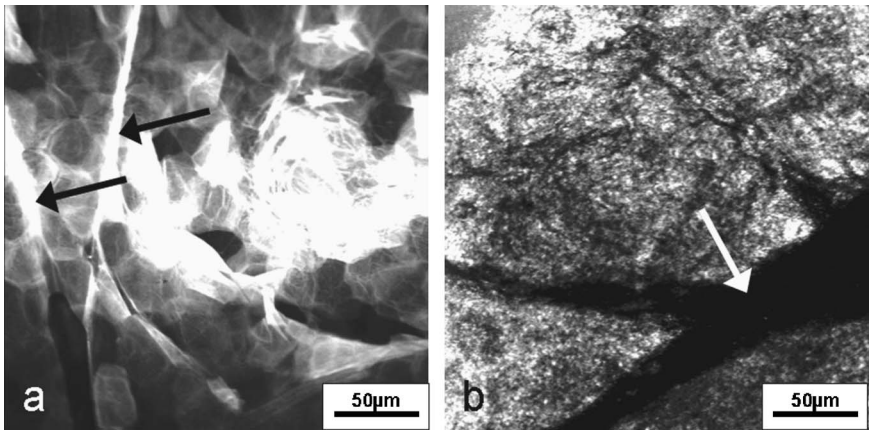

Fig. 1 The horny layer (stratum corneum) obtained by confocal microscopic imaging. (a) The fluorescence imaging showed single corneocytes and bright skin furrows (black arrows). (b) Compared to, in reflectance mode, dark furrows (white arrow) and "islands" of corneocytes were detectable.

face. Altogether, the aspect of normal living skin in contrast to its pathogenic state can be exposed.[4,10,11]

To date, two different modes of *in vivo* confocal imaging are established in dermatology: the reflectance and the fluorescence modes. Reflectance mode microscopic scanning achieves contrast by utilizing the inherent refractive index properties of the various cellular microstructures. The radiated laser beam is scattered irregularly by the heterogeneous cutaneous components. Backscattered in-focus signals are captured, transmitted to a spectrometer, and submitted for visualization. Consequently, the contrast is associated with the naturally occurring components in skin tissue. Usually, a laser light with near-infrared (near-IR) wavelengths is used for *in vivo* reflectance mode measurements. Thereby, skin components such as melanin or keratin have a high refractive index. They strongly backscatter near-IR light, which particularly grants a bright imaging contrast from cells containing melanin or keratin (e.g., melanocytes from the dermoepidermal junction or corneocytes from the superficial horny layer).[12] Several publications proclaim accurate results and great effectiveness by using this method *in vivo*. Histometric analyses of normal skin parameters,[2] as well as comparisons between the healthy aspect and the pathological state of living human skin were performed for diagnostical purposes.[4] For instance, a multicenter study was carried out, presenting a very high sensitivity and specificity of reflectance mode CSLM for noninvasive diagnosis of basal cell carcinoma.[13]

In contrast, only limited reports of fluorescence confocal microscopy exist in the field of clinical diagnosis.[11] CSLM in fluorescent mode relies on the differential distribution of an exogenous fluorescent dye to produce contrast. The dye has to be administered before measuring. Subsequently, a laser light source, at an appropriate wavelength, is used to selectively excite the applied agent. The emitted fluorescence signal is detected simultaneously and exploited to create an imaging contrast. In our laboratories, for fluorescence mode imaging, we used visible illumination, a blue laser light. This allows a greater selection and margin of fluorescent contrast dyes.[14] Differing from near-IR wavelengths, melanin absorbs light in the blue wavelength range. This means that cells containing the dark biological pigment, such as basal cells and melanocytes, as well as pigmented lesion, image darkly. To date, *in vivo* fluorescent confocal microscopy has been used for real-time investigations of skin structures and penetration processes of different pharmacologic and cosmetic substances, allowing new predictions and predications on human skin physiology.[3,15]

Both methods of CSLM, alone, have shown a great potential in dermatology. Although they are quite similar in structure and function, their scope of application is still separated and attached to definite scientific tasks: as mentioned above, the reflectance mode is predominantly used for morphological studies and in the clinical diagnostical field, whereas the fluorescence mode is suited to penetration research and laboratory concerns. In their privileged field of application, both are widely accepted and established as useful investigation tools. To date, however, these promising imaging modes have not been compared directly by combining and testing them *in vivo* in humans. Thereby, both scanning methods are consistent and could supplement each other to extract additional information from the state of the tissue.

The aim of the present study was to oppose the fluorescence and reflectance mode of CSLM in dermatology. We demonstrate advantages and disadvantages by comparing their

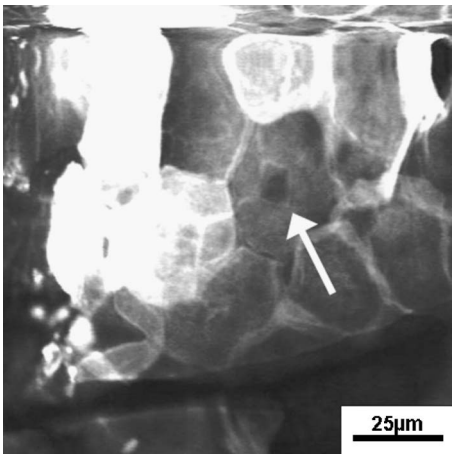

Fig. 2 Remnants of apoptotic nuclei. Seen in the stratum corneum, especially in the fluorescent CSLM.

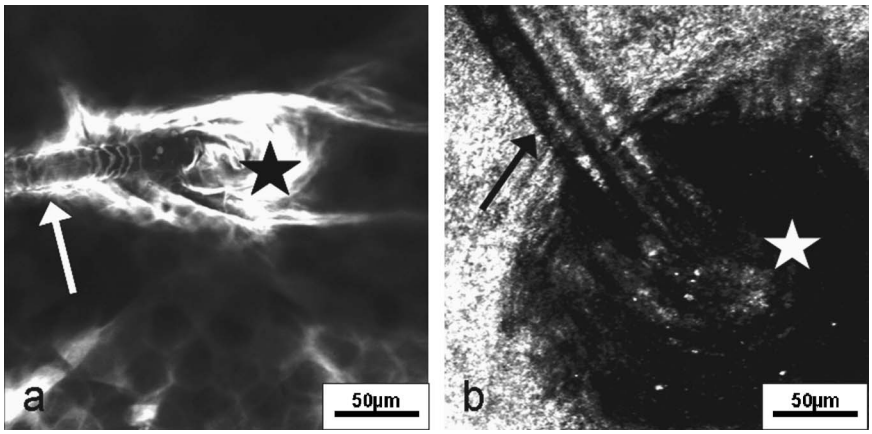

Fig. 3 Confocal images of hair follicles differ in reflectance and fluorescence mode. (a) In the fluorescence mode the superficial hair follicles were perceived as bright shining ring structures (star), whereas (b) in reflectance confocal microscopy they image darkly (star).

practical implementation and by interpreting their images. The pictures were obtained from different skin sites and depths from healthy human skin. Both optical noninvasive techniques alone, or in combination, launch new prospects and possibilities in the medical investigative field.

2 Materials and Methods

2.1 *Volunteers*

The experiments were carried out in 8 healthy volunteers (aged between 26–69 years) on different skin regions including the scalp, lower arm, hand, and lower leg. A compound nevus and skin annexes, such as sweat glands and hair follicles, were also studied on a number of the volunteers. All volunteers were Caucasians, with skin types ranging from I to III. They had been informed about the aims and risks of the study and had given their informed written consent. Ethics approval had been granted by the University Hospital Charité Ethics Committee, Berlin, Germany.

2.2 *Material*

Imaging was realized by using two different commercial confocal systems. For the reflectance mode, a dermatological near-IR laser microscope (VivaScope 1500®, Lucid Inc, Henrietta, NY, USA) was used, whereas the fluorescence-imaging instrument worked with a laser source at visible illumination wavelengths (Stratum®, OptiScan Ltd., Melbourne, Australia).

Generally, longer wavelengths show a deeper penetration into the skin, but they also provide less lateral resolutions.[4] By using the near-IR VivaScope 1500®-instrument at an illumination wavelength of 830 nm, it is possible to observe normal skin up to 250–300 μm in depth. In comparison, the illumination source of the Stratum® is a 488-nm-single-line argon ion laser. Its blue laser light is limited in depth in the region of 150 μm in human skin tissue. Therefore, both modes are sufficient for imaging the whole epidermis and parts of the upper dermis. Nevertheless, the illumination of the blue Ar^+-laser is confined to the papillary dermis, whereas the near-IR laser reaches additional underlying upper reticular dermis. Thereby, the VivaScope 1500® achieves an optical lateral resolution of ~ 2 μm and axial resolution of >5 μm (optical section thickness), whereas the Stratum® shows an optical resolution of ≤ 1 μm laterally and ≤ 3 μm axially. The single test field of view of the reflectance microscope VivaScope 1500® is 500 $\times 500$ μm, the pixel acquisition rate is fixed at 1000 $\times 1000$ pixels. We used a 30$\times$ water immersion objective lens with a numerical aperture of 0.9 for our clinical investigations. The intensity of the backscattered light is digitized by a light detector into an 8-bit gray scale for measurements. Additionally, confocal mosaics, composed of single fields of view, allow fast low-resolution examination of skin tissue in large optical skin excisions, enabling an immense overview and an easy finding and steering for single skin structures, e.g., hair follicle or papillae. The scanning frame rate is set at approximately 10 frames per second, allowing static pictures to be produced, as well as movies of dynamic events (e.g., blood flow).[16] In contrast, the field of view of the Stratum® is smaller at 250×250 μm. The customized objective lens in the scanner focuses the laser beam within 0.5 mm from the outer surface of the scanner tip at an effective numerical aperture of approximately 0.5.[11] The detection system works with a high-sensitivity, multi-alkali photocathode photomultiplier tube detector combined with a fluorescence barrier filter with a detection bandwidth of 505–585 nm. The fluorescence emission's intensity at the photo detector is measured and digitized into a 12-bit gray scale for display. The digital image resolution is variable on account of the scan speed. For highest resolution (1024×1024 pixels/image) we worked with a scanning frame rate of 1.4 s/frame.[14]

Both *in vivo* confocal systems are classified as a Class 3A laser device and have been approved by the Food and Drug Administration (FDA).

2.3 *Methods*

Modest preparation procedures are required before measurements can be performed. The imaging skin site of the volunteers had to be prepared differently for both scanning modes: For the reflectance mode, water immersion lenses or water-based gels as immersion media are applied onto the skin surface. The reflectance index of water (1.33) is close to that of the epidermis (1.34).[4,10] This minimizes spherical aberrations and irregularities in refraction caused when the laser light

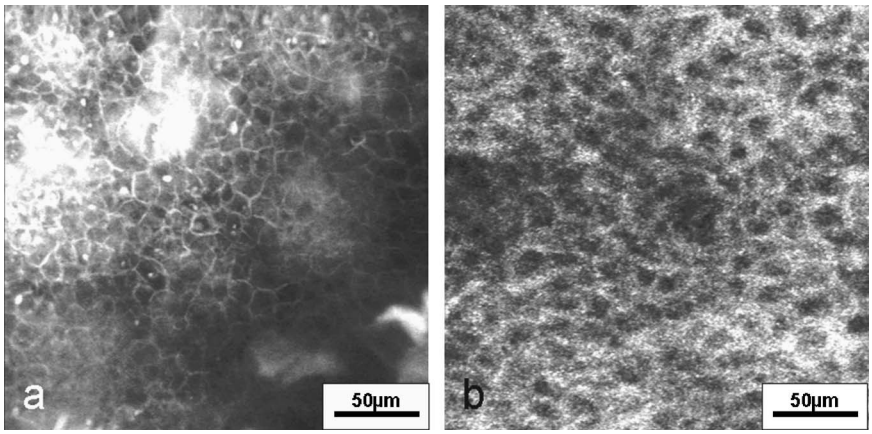

Fig. 4 Stratum granulosum. (a) Initially, dark nuclei and cytoplasm, and bright extracellular spaces were common in fluorescent images. (b) Dark oval nuclei and bright, grainy cytoplasm were suspicious in reflectance mode imaging.

passes the tissue–air interface. Consequently, the preparation procedures of the reflectance CSLM is entirely noninvasive and cannot cause any damage to tissues.

The fluorescent mode is more invasive. It needs the compulsory application of a fluorescent dye before confocal imaging can be accomplished. In our case, a 0.2% solution of sodium fluorescein (Fluorescein SE Thilo, Producer Alcon, France) was used as contrast agent for labelling human skin structures. Sodium fluorescein has a maximum absorption rate at a wavelength around $\lambda = 480$ nm, and the fluorescence emission is peaking at about $\lambda = 520$ nm. For imaging subsurface cutaneous details, the penetration obstacle of the superficial horny layer was avoided by the insertion of a thin hypodermic syringe.[11] An intradermal injection of approximately 20–30 μl of the dye's solution was performed. One drop of sodium fluorescein was also applied topically to exactly the same site for adequate labelling skin surface structures. Therefore, scanning of multiple skin areas needs repetition of the staining, implying a greater application effort than reflectance imaging.

Ultimately, we studied the depictive representations from both *in vivo* confocal imaging modes. The morphologic and cellular features were differently emphasized in normal human skin. Typical images within the diverse epidermal layer were obtained and compared. Fluorescent images are shown on the left side, and reflectance images within the same depth localization are arranged on the right in Figs. 1, 3, 4, 6, and 7. Exceptions are Figs. 2 and 8, demonstrating fluorescent images, as well as Fig. 5, illustrating the pharmacokinetic process of fluorescein sodium solution.

3 Results

Generally, in order to interpret confocal images of normal skin, one must be familiar with the structure and appearance of human skin tissue seen by light microscopy. The obvious differences are that the confocal images received are in a horizontal view (*en face*), instead of the vertical sections that are normally obtained from routine histology, and also that they are gray-scaled.[4]

We demonstrate confocal images by starting from the skin surface and progressing deeper. The most superficial cell layer, the horny layer (stratum corneum), engendered a very bright contrast by using the reflectance mode (Fig. 1(b)). The

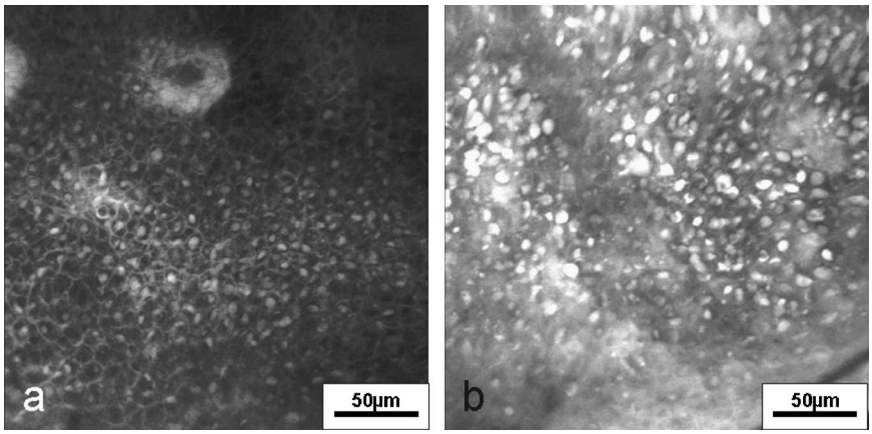

Fig. 5 Illustration of the kinetic distribution process of the fluorescence dye. Both figures show fluorescent confocal images in the same depth localization at different points of time. Initially, fluorescein was confined predominantly to the intercellular spaces (Figs. 4(a) and 6(a)). (a) The occurrence of increased nuclei indicated the dye's intracellular diffusion (bright spots). (b) Later on, the cytoplasm shines brightly.

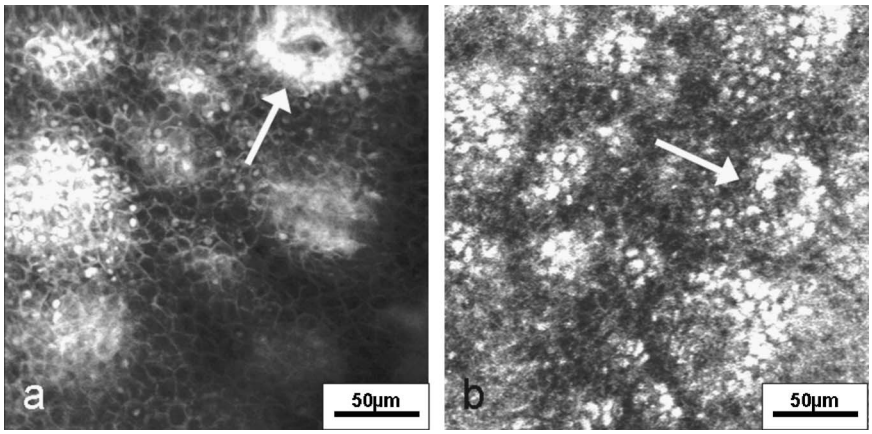

Fig. 6 Stratum spinosum. (a) In the fluorescence mode imaging, basal cells appeared dark and papillae bright. A capillary loop was hit, embedded in the hilltop of a papilla (arrow). (b) In contrast, reflectance CSLM showed bright melanin-containing basal cells besieging a grainy papilla (arrow).

high refractive difference between the keratin-containing stratum corneum (1.54) and the immersion medium (water at 1.33) resulted in a significant amount of back-scattered light.[4,10,12] Irregular asymmetric, horny "islands" separated by dark skin furrows and wrinkles are visible in Fig. 1(b) (white arrow). The cell borders can only be detected sporadically by reflectance CSLM. In fluorescent mode, the superficial corneocytes fluoresced immediately after application of the contrast dye (Fig. 1(a)). The images clearly and lucidly showed every single, polygonal, flattened, and anucleated corneocyte, 30–45 μm in diameter. These cells were surrounded by brightly shining skin folds caused by the admission of the fluorescent dye (Fig. 1(a), dark arrows). Remnants of apoptotic nuclei were noted in some cases (Fig. 2, white arrow).

Additionally, in both imaging modes, bright hair could often be seen (Fig. 3, arrows); whereby in the fluorescence mode, the superficial hair follicles were perceived as bright fluorescent ring structures (Fig. 3(a), star) as a result of accumulating topically applied fluorescein; they were contrasted darkly in the reflectance mode (Fig. 3(b), star). A similarity was perceived on sweat glands.

The next epidermal layer obtained was the stratum granulosum (Fig. 4). In both application modes, large keratinocytes (20–35 μm) were arranged in a cohesive array, constituting only a few cell layers.

Initially, in the fluorescence mode, the dye was confined to the extracellular spaces.[11] Cell borders were stained and fluoresced brightly (Fig. 4(a)). Cell size, shape, and morphology could clearly be recognized. The dark cytoplasm could not be distinguished from the unmarked cell nuclei in the first 5 minutes. Apart from the unlively stratum corneum, in all living epidermic skin, including stratum granulosum, a variation in the distribution of the fluorescence dye was observable *online*: the fluorescein sodium solution penetrated into an intracellular location, primarily labelling cell nuclei (Fig. 5(a), bright central spots), followed by the cytoplasm (Fig. 5(b)). This enables predications on the cell diffusion barrier and on the appearance of the nuclei (e.g., inflammatory cells occurred earlier). Imaging quality was peaking between 3 and 5 minutes after introduction of the contrast dye (extracellular distribution pattern), decreasing over time (which correlates with the intracellular release), with disappearance of the contrast after 25–40 minutes. Therefore, in opposition to the reflectance mode, fluorescence confocal imaging showed a dynamic process of the fluorescence dye's distribution, accentuating different cell structures at different time points.

On the contrary, the reflectance mode demonstrated "static" confocal images of the stratum granulosum (Fig. 4(b)). Keratinocytes were measurable, and the nuclei could be appreciated as dark central spots, surrounded by bright grainy cytoplasm. Here a precise delimitation of the cell membranes appears arduously.

The keratinocytes in the underlying epidermal layer, stratum spinosum, were smaller (10–15 μm) and arranged in a distinctive, tight honeycomb-pattern.[4,11] For the first time, acellular round "hilltops" of the papillary dermis (papillae) were tangential truncated (Fig. 6, white arrows), embedding dark capillary blood vessels. Small basal keratinocytes (7–12 μm) were circumferentially arranged around the papillae. As the focal plane was progressed in depth to the basal layer, these round papillae increased in size (Fig. 7, white arrows). The afferent and efferent capillary limbs could be observed as round structures within the papillae (Figs. 6 and 7).

Chiefly, at this depth zone, differences exist in the optical occurrences of the melanin-containing basal cells and of the papillary dermis. Frequently, in the fluorescence mode, cells of the basal layer showed darker cytoplasm compared to the epithelial cells of the other cutaneous layers (Fig. 8, arrows). The darkness of the basal cells was enhanced in individuals with darker pigmentation and in pigmented skin lesions, e.g., nevi. The collagen-fibres of the papillary dermis store fluorescein well. The papillae were observed as bright, homogeneous, acellular round buildings in the fluorescence mode (Figs. 6(a), 7(a), and 8). On the other hand, the reflectance images showed bright clusters of basal cells, surrounding significantly darker papillary dermis at the dermoepidermal junction (Figs. 6(b) and 7(b)). The reason for this finding is the high refractive index of melanin.[12,16] It provides a strong reflection for near-IR laser light, most excellently for imaging pigmented skin disorders, for instance, melanoma. The plasma of the blood vessels appeared dark in both methods; isolated tumbling blood cells were visible, especially in the reflectance mode.

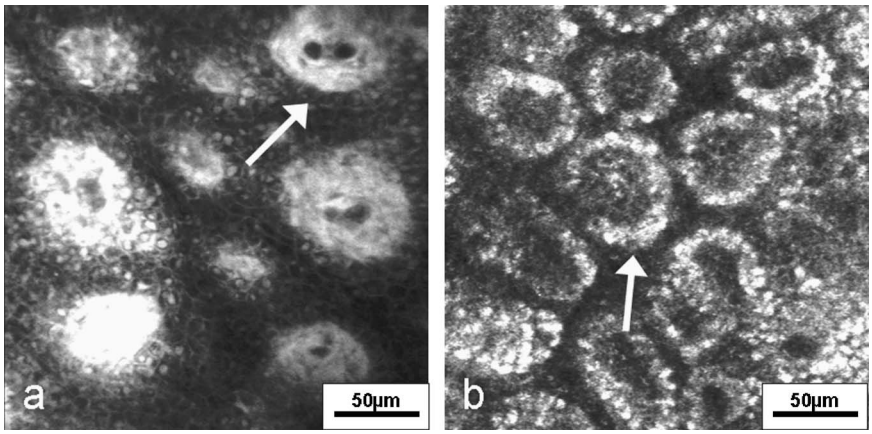

Fig. 7 Stratum basale/papillary dermis. (a) Fluorescent images showed bright and fibrous papillae, containing capillary limbs. (b) A different contrast-accentuation in the reflectance mode: basal cells were highlighted brightly, papillae shimmered grainy and darkly, and blood vessels were also seen black.

By shifting the depth of the focal plane further into the skin tissue, in both methods it was possible to trace down the rete ridges to the deepest basal keratinocytes. In skin of thin epidermis, the fluorescent mode enabled scanning of the papillary dermis. In the reflectance mode, imaging of the upper reticular dermis was possible. Here, the resolution of the images was merely of poor quality and only minor expressive.

4 Discussion

Compared with light microscopy, the advantages of confocal imaging include the absence of biopsy specimens, accordingly, no pain or reduced pain, immediate results, and an easy scanning of multiple areas, without an intensive preparation procedure. Observation of living cells in their native habitat and follow-up of exactly the same skin site can be performed, as no tissue is removed. Restrictions consist of low imaging depths and the unaccustomed horizontal view on the histoimages, which are different from the typical vertical histosections used in conventional light microscopy.[4,10,11]

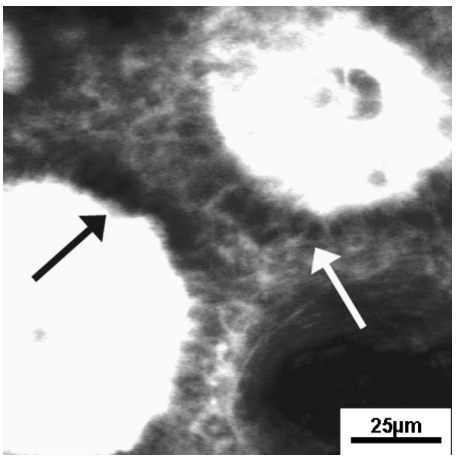

Fig. 8 In the fluorescent mode melanin-containing basal cells were observed dark (white and dark arrows). They surround bright papillae, including dark blood vessels.

In the last years, two different application modes of CSLM could be established in dermatological practice: the reflectance and the fluorescence modes. As shown above, both allow reliable predictions in skin morphology. Advantages of near-IR reflectance mode imaging involve deeper scan values, noninvasive usage, as well as a strongly backscattered contrast of melanin-containing cells.[12,16] These abilities promise an intensified implementation of reflectance CSLM in the field of skin cancer investigations, diagnosis, and treatment control, especially in the case of melanoma.[17,18]

The combination of *in vivo* CSLM and an exogenous fluorescent dye enables scanning of the whole epidermis to a depth of about 150 μm using visible illumination wavelengths. In contrast to the reflectance mode, the dynamic distribution of the contrast dye can be observed over a designated period of time. Therefore, the pharmacokinetic processes provide additional functional information on the state of the tissue. Changes in the dye's diffusion can be exploited for grading skin disorders.[11] This implies, apart from the use as a penetration research instrument, that fluorescence confocal imaging will be a valuable tool in the study and diagnosis of diseases of the epidermis and the upper dermis. However, further investigations are needed to ensure the potential of fluorescence mode CSLM in investigative dermatology. Furthermore, supplementing staining protocols need to be comprehended and established. For instance, progress in the capability to target subcellular cutaneous structures with specific fluorescent-labelled antibodies would exceedingly extend the role of fluorescent CSLM in clinical diagnostics.[19]

In conclusion, both confocal methods show strengths and weaknesses in the imaging of living human skin tissue. To date, a feasible technical linkage in one aperture, promising amplified predictions and information, was not realized. Both confocal microscopic modes and a combination of both provide auspicious prospects for the investigative future in dermatology.

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