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Multiphoton Laser Scanning Microscopy—A Novel Diagnostic Method for Superficial Skin Cancers

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The increasing incidence of skin cancer and the importance of early diagnosis is a challenge, which requires the development of reliable, cost-effective, noninvasive, diagnostic techniques. Several such methods based on optical imaging techniques are available and currently being investigated. A novel method in this field is multiphoton laser scanning microscopy (MPLSM). This technique is based on the nonlinear process of 2-photon excitation of endogenous fluorophores, which can be used to acquire horizontal optical sectioning of intact biological tissue samples. When studying human skin, MPLSM provides high-resolution fluorescence imaging, allowing visualization of cellular and subcellular structures of the epidermis and upper dermis. This review covers the application of MPLSM as a diagnostic tool for superficial skin cancers, such as basal cell carcinomas, squamous cell carcinoma in situ, and melanomas. MPLSM has also been applied in other research areas related to skin, which will be mentioned briefly. The morphologic features observed in MPLSM images of skin tumors are comparable to traditional histopathology. Safety issues, limitations, and further improvements are discussed. Although further investigations are required to make MPLSM a mainstream clinical diagnostic tool, MPLSM has the potential of becoming a noninvasive, bedside, histopathologic technique for the diagnosis of superficial skin cancers.

KEYWORDS multiphoton laser scanning microscopy, fluorescence, skin cancer, optical biopsy, histopathology

The rising incidence of melanoma and nonmelanoma skin cancer, for example, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), is a major health concern in countries with a fair-skinned population.[1-3] The routine diagnostic procedure for these tumors is generally visual inspection followed by a biopsy or excision and subsequent histopathological examination. This is an invasive procedure associated with delayed diagnosis and costly human resources. Recently, several tools facilitating noninvasive diagnostics have emerged, most of them based on optical imaging. The simplest one is dermoscopy, which is widely used today. With dermoscopy, subsurface anatomic structures of the epidermis and papillary dermis of skin lesions are visualized using a magnifying lens and transilluminating light.[4-6] Another technique is fluorescence diagnosis, which is based on tumor-selective fluorescence after application of aminolaevulinic acid.[7,8] Fluorescence diagnosis is mainly used for demarcation of BCCs and has not gained clinical acceptance.

The major drawbacks of dermoscopy and fluorescence diagnosis are that these techniques only yield superficial and macroscopic information. Optical coherence tomography (OCT), by contrast, can image tissue morphology down to a depth of approximately 2 mm. OCT has been applied in several medical fields, especially in ophthalmology,[9] and has recently been introduced for skin cancer diagnostics.[10,11] OCT is a laser scanning technique in which the data resembles an ultrasound image with slightly better resolution (axial resolution = 8 μm, lateral resolution = 24 μm), but still too low for visualizing cell morphology.[11] To study morphologic changes on the cellular level, optical microscopy with higher resolution must be involved. Unfortunately, as biological tissue is not transparent in the visible part of the light spectrum, conventional optical microscopy cannot reach deeper than a few cell layers. Thus, a better choice is optical microscopy based on near-infrared (NIR) light, because the absorption and scattering probability in biological tissue is lower in this part of the spectrum.

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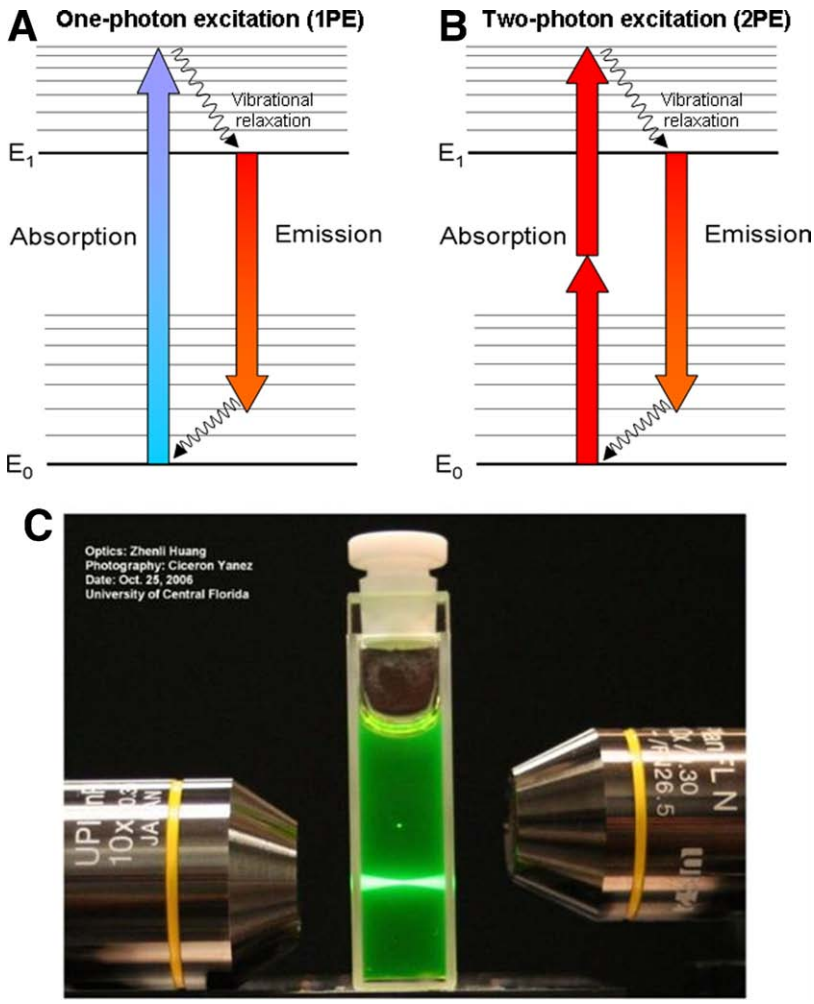

Figure 1 Schematic drawing illustrating the processes of (A) 1-photon and (B) 2-photon excitation. Absorption of light leads to energy transition from the molecular ground state, E_0, to a higher electronic energy state, E_1. The energy of the emitted light is the same irrespective of the type of excitation. (C) Photograph showing the fluorescence arising from 1- and 2-photon excitation in a fluorescent dye (fluorescein) solution. (Photograph by the Belfield Research Group, University of Central Florida and published with the kind permission from Prof. Kevin D. Belfield.)

One such technique is reflectance-mode confocal laser scanning microscopy (RCLSM), where images of tissue morphology are constructed by detecting the back-reflected NIR light using confocal microscopy technology.[12] The resolution in RCLSM is approximately 0.5-1.0 μm in the lateral direction and 3-5 μm in the axial direction, giving the possibility of observing cellular structures down to the upper dermis. Several groups have published promising results in applying RCLSM for diagnosis of skin cancer and instruments for clinical use are available.[13-15] Another such imaging technique is multiphoton laser scanning microscopy (MPLSM), which is the topic of this review. Similar to RCLSM, this is a NIR laser scanning microscopy technique, but instead of recording the backscattered light, MPLSM is based on nonlinear processes. Two-photon excitation (2PE) of fluorescent substances is the most commonly used nonlinear process. This process was predicted already in 1931 by Maria Göppert-Meyer[16] but was not used in microscopy until 1990.[17] There are several endogenous fluorophores in skin tissue, for example, NADH, keratin, melanin, collagen, elastin, which can be excited by 2PE.[18,19] Thus, tissue morphology can be visualized on the cellular and subcellular level down to a maximum imaging depth of around 200 μm. The resolution is slightly better than for RCLSM, around 0.5 μm and 1-2 μm in the lateral and axial directions.[20] MPLSM has the potential of becoming a bedside noninvasive imaging technique and has been introduced for skin cancer diagnosis.[21-24] There is even a commercially available instrument (DermaInspect, JenLab, Germany) that has been approved for clinical use.[20,25] The aim of the present paper is to give an overview of the recent reports on the use of MPLSM for skin cancer diagnostics. We also briefly describe the technical aspects of MPLSM, other applications, safety issues, limitations, and future improvements of the technique.

Technical Aspects

The main difference between MPLSM and conventional fluorescence confocal laser scanning microscopy is the excitation process, which in MPLSM is based on nonlinear optical

processes.[26] As already mentioned, the most commonly used nonlinear process for studies of biological tissue is 2PE (ie, the simultaneous absorption of 2 photons, which precedes the emission of the fluorescence). Figure 1A and B illustrates 1-photon excitation (1PE) and 2PE processes of a fluorophore. In both cases, the fluorophore is excited from its electronic ground state (E_0), to a higher electronic state (E_1), by the absorption of light. In 1PE, the excitation wavelength matches the energy gap between the 2 electronic energy states. In 2PE, 2 photons with less energy (ie, longer wavelength) are absorbed simultaneously and their combined energy can excite the fluorophore to E_1. The excitation light normally lies in the ultraviolet to green region for 1PE, while the excitation light lies in the NIR range for 2PE, which enables deeper light penetration.

Because 2PE is a nonlinear process, where 2 photons have to be absorbed simultaneously (within 10^{-18} to 10^{-16} s), its probability of occurring is extremely low. This means that high-photon density (high laser power) is necessary. This aspect is illustrated in Fig. 1C, in which a homogenous solution of a fluorescent dye is excited by both 1PE and 2PE. The 1PE fluorescence using 380-nm light focused by the objective to the left is observed as a double cone of green light, with the beam waist at the focal plane. In contrast, the 2PE fluorescence using 760-nm light focused by the objective to the right is exclusively seen as a small spot present at the focal point, which is the only location with sufficient photon density. Therefore, photobleaching is greatly reduced compared to 1PE and the fluorescence detection can be more efficient because there is no need to exclude out-of-focus emission. To obtain the high-photon densities necessary for the 2PE process, while keeping the average power sufficiently low in order not to harm the tissue, the excitation light needs to be concentrated in both space and time. Therefore, a laser with short pulses, usually a femtosecond titanium-sapphire laser, is used for MPLSM.

The 2PE fluorescence can be combined with other nonlinear processes, for example, second harmonic generation (SHG) and fluorescence life-time imaging. These techniques have been described elsewhere[18,26] and will not be discussed further here because most of the studies of MPLSM for skin cancer diagnostics mainly focus on 2PE fluorescence.

Areas of Research

One of the first studies published on MPLSM on human skin is from Masters et al in 1997, where they visualize the cellular structures of the epidermis and upper dermis, by placing the forearm of one of the authors into their microscope.[27] In addition to studying the skin morphology, the authors also present spectral measurements of skin autofluorescence, supporting that NAD(P)H is the primary source of the autofluorescence in the epidermis. Because the imaging of endogenous fluorophores offers the possibility of noninvasive tissue histology, several authors have continued the quest of developing real-time noninvasive microscopy techniques for skin diagnosis. A recent review covers MPLSM, SHG, and fluorescence lifetime imaging for investigations of skin in a more general manner, such as skin morphology, monitoring skin damage, molecular penetration, and skin transport.[18] Thus, our present review focuses on MPLSM as a diagnostic method for superficial skin cancers. Other applications are only mentioned briefly. MPLSM has been used to study a variety of skin diseases, for example, psoriasis, nevi, melanomas, BCC, and dermatomycosis,[20,21] but most of the studies have been performed on occasional biopsies ex vivo. Only a few papers investigating the diagnostic ability of MPLSM in a more rigorous manner exist.

Most of the publications on MPLSM on human skin in vivo deal with normal human skin on healthy volunteers.[20,27,28] In Fig. 2A, a typical example of the morphologic features observed at different tissue depths in normal skin are shown. The stratum corneum (SC), mainly consisting of keratin, is highly fluorescent and is visible as large, flattened, anucleated corneocytes. Below the SC, the cells are characterized by fluorescent cellular cytoplasms surrounding nonfluorescent nuclei. The epidermal cells are homogeneously distributed and separated by thin nonfluorescent intercellular spaces. The cells of the stratum granulosum (SG) generally exhibit a weaker fluorescence than other epidermal cells, resulting in a lower overall fluorescent signal from the SG layer. The cytoplasmic autofluorescence increases with epidermal depth, while a gradual decrease in cell size is noted from the stratum spinosum (SS) down to the stratum basale (SB). The apical poles of the cuboidal cells of the SB are highly fluorescent due to the high melanin content. Going deeper, rounded areas appear corresponding to the tips of the dermal papillae (DP). Within these areas, bright and highly fluorescent filaments are visible corresponding to elastin and collagen fibers. Going deeper (100-150 μm), only connective tissue fibers surrounded by nonfluorescent stroma are visible.[24]

Our group has performed a clinical study on MPLSM where lesions from 14 patients with BCC or SCC in situ (SCCIS) were investigated ex vivo.[24] We were able to verify that traditional histopathologic features could indeed be observed using MPLSM. SCCIS specimens (Fig. 2B) showed an abnormally thick SC with hyperkeratosis in the form of fluorescent nuclei compartments within the corneocytes and, in some cases, large rounded bundles of keratin, corresponding to so-called keratin pearls. The keratinocytes within the SG, SS, and SB were irregularly distributed with widened intercellular spaces and pleomorphic and multinucleated cells. Dyskeratotic keratinocytes were observed with brighter cytoplasmic fluorescence compared to surrounding cells in the SG and SS. Several lesions presented speckled perinuclear fluorescence in the SG and/or SS, a feature also observed in SCC in a hamster cheek pouch model ex vivo.[29] The superficial BCC (sBCC) specimens (Fig. 2C) presented epidermal thickening and hyperkeratosis, which made the dermis difficult to be visualized. Tumor cells were monomorphous and disposed in palisade in the periphery. In 1 lesion, keratinocytes presented elongated nuclei and cytoplasms, a characteristic known as nuclei polarization.[14,30] Typical nodular

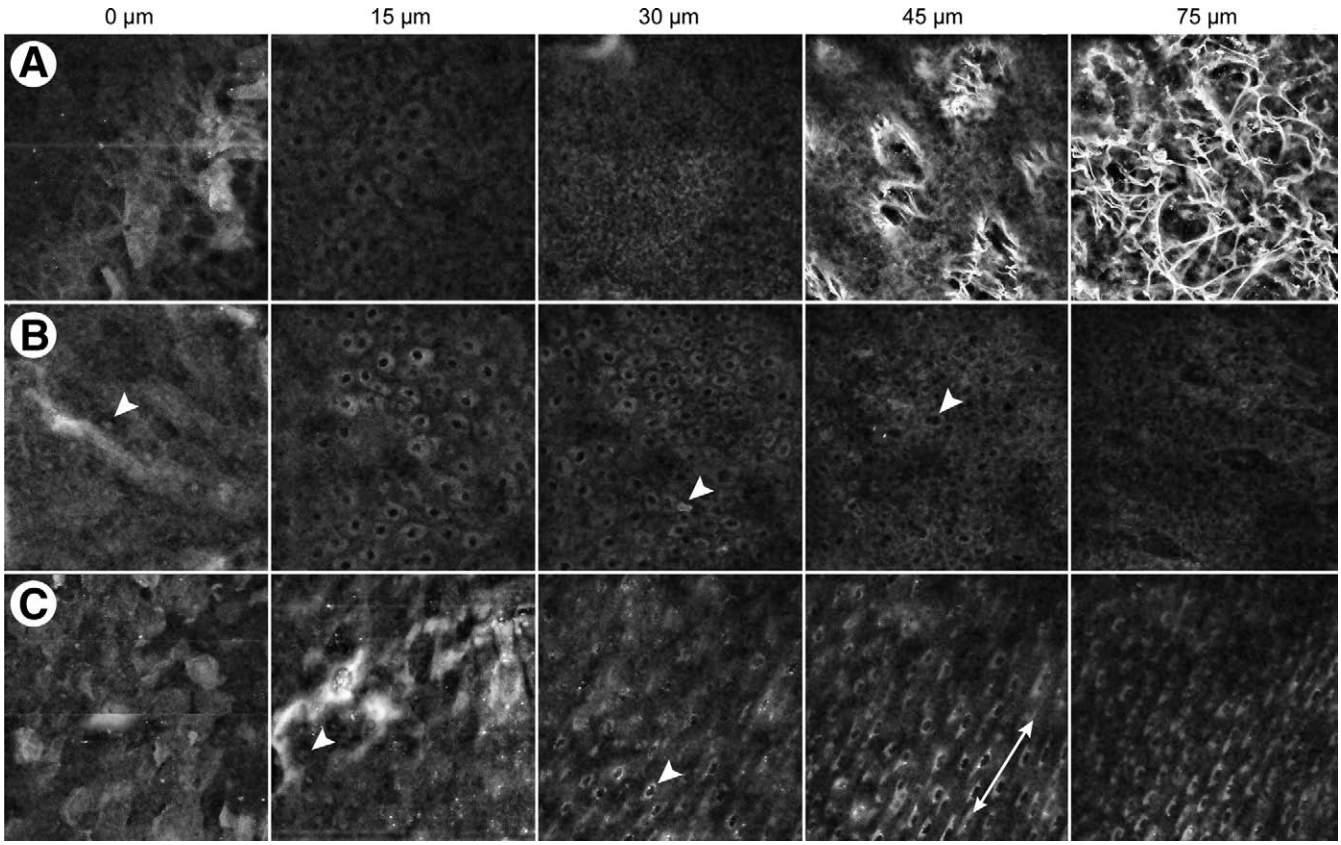

Figure 2 MPLSM images of (A) normal skin, (B) a SCCIS specimen, and (C) a sBCC specimen. (A) Normal skin showing the SC (0 μm), the SS (15 μm), the SB (30 μm), the DP (45 μm), and the dermis (75 μm). (B) A SCCIS sample presenting fluorescent nuclei compartments in the SC (0 μm, arrow), widened intercellular spaces, and a loss of cell polarity in the SG (15 μm), a dyskeratotic cell in the SS (30 μm, arrow), a multinucleated cell among several atypical cells of the SS/SB (45 μm, arrow), and the DP (75 μm). (C) A sBCC specimen with corneocytes of normal appearance (0 μm), presenting fluorescent nuclei compartments at 15 μm (arrow), speckled perinuclear fluorescence among the keratinocytes at 30 μm (arrow), and elongated polarized nuclei at 45 μm (double-pointed arrow) and 75 μm. Figure reused with permission from the *Journal of Investigative Dermatology*.[24]

nests of tumor cells were only found in 1 nodular BCC (nBCC) lesion.[24] These nests contained basal cells with large, oval nuclei, little cytoplasm, and peripheral palisading without contiguity with the overlying epidermis of normal appearance. However, because the imaging depth was limited to around 150 μm, imaging of nBCCs was found to be difficult.

The most extensive clinical MPLSM study, so far, comprised 83 patients with suspected melanoma.[22] Microscopy was performed both in vivo and ex vivo, and 4 different observers evaluated the images. The results showed distinct morphologic differences in melanomas compared with melanocytic nevi. Six characteristic features were observed: architectural disarray within the epidermis, pleomorphic cells, poorly defined keratinocyte cell borders, dendritic cells, large intercellular distance, and ascending melanocytes. Among these, the first 4 features seem to provide the most significant diagnostic criteria. Overall sensitivity values of 84% (range: 71%-95%) and overall specificity of 76% (range: 69%-97%) were achieved for diagnostic classification. Interestingly, higher sensitivity was achieved in MPLSM images acquired ex vivo as surface irregularities and motion artifacts were eliminated and a higher laser power could be used, which provided better image quality.

Besides cell morphology, spectral information also provides valuable information, which can be used to discriminate between tumor and perilesional tissue.[21,23] The ratio between the autofluorescence signal and the SHG signal has been shown to be elevated in tumor stroma of nBCCs.[23] These results are based on MPLSM images on thin tissue sections of 3 human tumors.

Other Applications

Instead of using the weak endogenous fluorescence of the skin, fluorescent probes can be used. For example, MPLSM could be included in conventional histopathology using fluorescently labeled antibodies to study tissue morphology.[31] Also, the combination of MPLSM and polarization imaging of stained skin specimens has been discussed.[32] In addition to skin cancer diagnostics, MPLSM has also shown to be a valuable tool for studying skin aging,[33] monitoring wound heal-

ing[34] and pH gradients in skin,[35] as well as topical drug delivery.[36,37]

Safety Issues, Limitations, and Future Improvements

Before MPLSM can be accepted as a mainstream clinical diagnostic tool, safety issues have to be properly addressed; however, so far not much has been done on the topic. All endogenous chromophores, except for melanin, have low 1-photon absorbance in the NIR range. Because NIR is used in MPLSM and the average power is kept low, it is expected that a major part of eventual tissue damage should originate from nonlinear effects, with the exception of melanin-related tissue damage. Indeed, melanin has been reported to be involved in 1PEs causing tissue damage at the dermoepidermal junction.[38] The nonlinear effects, by contrast, give rise to 2- and 3-photon absorption by, for example, DNA and NADH. The photochemical effects after femtosecond-laser irradiation have been shown to be similar to the DNA damage obtained after ultraviolet exposure.[39] Interestingly, in this study, the authors used the clinically approved DermaInspect device to induce the damage. To avoid unwanted tissue damage, the safe laser power range for MPLSM has to be properly investigated.

Apart from the safety issues, MPLSM currently has some other limitations as a diagnostic tool. The imaging depth is insufficient to study thick, invasively growing tumors, for example, nBCCs.[24] Also, when imaging SCC, the imaging of dermal structures becomes limited by the presence of hyperkeratosis, parakeratosis, and the acanthotic epidermis. To image deeper lying structures, laser power must be increased, which may lead to thermal damage of the tissue.[38] Thus, it is important to improve the technique so that dermal stroma can be properly investigated at safe laser powers. This could possibly be done with so-called optical clearing agents, for example, glycerol, propylene glycol, and glucose, which reduce the scattering properties in tissue.[40] However, it is unclear if or how these agents may affect tissue morphology.

Another issue is the time required to scan the tissue (10-15 min).[22] If applied in a clinical setting, MPLSM must be faster to gain acceptance and avoid distortion of the images due to movements made by the patient. Furthermore, many tumors require sampling of several areas, which adds to the time consumed. High-speed MPLSM has been investigated for cytometry,[41] but the authors stress the challenge of imaging human skin with a high-turbidity and low-autofluorescence signal at high speed.

Another future challenge is to miniaturize the equipment to allow for imaging of difficult-to-reach skin areas with surface irregularities and intrabody imaging. It seems possible to perform MPLSM using objectives as small as 2 mm in diameter,[42] but further work is necessary to obtain true microendoscopes based on MPLSM technology. Another important aspect is improving the axial resolution in MPLSM. For this, it is necessary to correct for the varying optical properties of human skin.[43] In addition, it is possible to perform image analysis to speed up the process of interpreting the MPLSM data.[44]

The final aspect is the cost of the MPLSM device, which presently is very high, due to the cost of the advanced lasers required. However, as the development of low-cost lasers for MPLSM is currently progressing,[45] we have hopes that the cost for such systems will be significantly reduced in the future.

Conclusion

This review covers the most important findings in MPLSM studies of nonmelanoma skin cancers and melanoma. In conclusion, MPLSM is a promising imaging technique that potentially could allow for bedside, noninvasive, histopathologic diagnosis of skin tumors and other skin diseases in the future. However, several limitations must be surpassed for this to be possible and, until then, routine histopathology remains the gold standard in the diagnosis of skin cancer.

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