MULTIPHOTON MICROSCOPY FOR NON-INVASIVE OPTICAL BIOPSY OF HUMAN SKIN

MULTIPHOTON MICROSCOPY – INSTRUMENT OPTIC NON-INVAZIV DE EVALUARE A PIELII

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Abstract

Multiphoton microscopy (MPM) is a laser scanning microscopy technique that can provide high resolution, label-free images of living tissues in their native environment that closely resembles the histological sections. In skin, several endogenous components can be visualized, including reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), keratin, melanin, collagen and elastin fibers. The recent development of MPM-based clinical tomographs advanced its practical utility, accelerated its applications and generated increased interest, particularly in the dermatology field. This review summarizes the most recent applications of in vivo MPM imaging in dermatology. We also discuss the challenges of implementing this technology into clinical practice.

Keywords:

multiphoton microscopy dermatology, non-invasive imaging skin cancer, non invasive optical biopsy, melanoma, basal cell carcinoma

Rezumat

Microscopia multifoton reprezintă o metodă de imagistică bazată pe scanare laser care produce imagini de înaltă rezoluție ale țesuturilor vii, în mediul lor natural, imagini asemănătoare secțiunilor histologice. Metoda nu necesită substanță de contrast și imaginile sunt obținute prin detectarea semnalului produs de diferite componente ale pielii (reduced nicotinamide adenine dinucleotide - NADH, flavin adenine dinucleotide - FAD, keratina, melanina, colagen, elastina) în urma excitării laser. Dezvoltarea recentă a tehnologiei de tip tomografie bazată pe microscopia multifoton a dus la îmbunătățirea metodei și la lărgirea spectrului de aplicații, generând interes în special în domeniul dermatologiei. Lucrarea de față include o sintetizare a celor mai recente aplicații în dermatologie ale microscopiei multifoton in vivo. Sunt de asemenea, discutate principalele provocări legate de implementarea acestei tehnologii în clinică.

Cuvinte-cheie:

microscopie multifoton, dermatologie, diagnosticare non-invazivă cancer de piele, biopsie optică noninvazivă, melanom, carcinom bazocelular

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Introduction

Microscopic evaluation of skin is required in many areas of dermatology such as diagnosing skin diseases, assessing the effects of cosmetic treatments or understanding the skin tissue functionality. Skin is thick and it scatters light strongly. Also, unstained tissue has very little contrast. The only way to image it with conventional optical microscopy is to slice it upon fixation, mount the slices on microscope slides, stain the slices on each slide and analyze them by using the standard optical microscope. This technique has two main limitations: 1. It is time consuming and it involves many steps in the process that can be prone to errors. 2. The tissue needs to be removed from its natural environment; while the overall tissue morphology is preserved during the slicing and staining process, the main features related to tissue functionality such as metabolism, are lost.

In recent years, optical technologies based on in vivo laser scanning microscopy have been developed to address these limitations. This method utilizes focused illumination of a laser beam to provide access to the sub-micron scale and raster-scanning across the sample to create high-resolution images. Each image plane (about 2μm thick) forms an optical section and can be used in reconstructing a 3D-view of the skin by scanning at multiple depths.

How laser scanning microscopy (LSM) can overcome high scattering and low contrast of unstained tissue is an area of current research. The scattering limitation has been solved only partially. Using a laser beam as a light source allows for wavelength selection that affects the scattering process. Therefore, longer wavelengths are scattered less than shorter wavelengths by a turbid medium such as skin. Infrared wavelengths are particularly used by LSM techniques for this reason. Nevertheless, limited depth penetration in skin due to light scattering remains a major limitation of these methods techniques and a permanent challenge that is being addressed as the technology advances.

Image contrast of unstained skin tissue, on the other hand, has been improved substantially and is based on different mechanisms corresponding to particular LSM techniques. Among these, reflectance confocal microscopy (RCM) and multiphoton microscopy (MPM) have been the most widely used in research studies related to applications in dermatology. RCM contrast is based on variations in tissue refractive index, which provide gray scale images with sub-micron resolution. MPM contrast in skin is derived from second harmonic generation (SHG) of collagen and two-photon excited fluorescence (TPEF) of tissue components such as the co-factors NADH and FAD, elastin, keratin, and melanin.

Due to its dual contrast mechanism, MPM provides dual-color images that distinguish cellular features from the extra-cellular matrix. MPM imaging is unique among other optical imaging technologies in that it provides 3D, near real-time sub-micron resolved label-free images of living tissues in their native environment with contrast that closely resembles the histological sections dermatopathologists use for diagnosis. MPM technology has been translated into clinical settings through the development of the MPM-based clinical tomographs, DermaInspect [1] and MPTflex by JenLab GmbH (Jena, Germany). This review summarizes the results of the most recent clinical studies in the dermatology field, performed by employing either the DermaInspect or the MPTflex tomographs.

Figure 1. MPM-based clinical tomograph MPTflex

MPM technology and translation into the clinic

The first experiments using SHG and TPEF laser scanning microscopy have been performed in 1986 [2] and 1990 [3], respectively. The first experiments on *in vivo* TPEF imaging of human skin were performed at MIT in the late 1990s [4-6]. TPEF and SHG imaging techniques were combined in a single MPM-based tomograph (DermaInspect) for *in vivo* skin imaging, at the University of Jena in 2003 (1). This system has been later developed by JenLab, GmbH (Jena, Germany) and CE-marked for clinical use along with its most advanced version MPTflex (Figure 1), a compact, portable device that features an articulated arm to allow imaging almost any region of the body. The system consists of a compact, Ti: Sapphire femtosecond laser, an articulated arm with near-infrared optics, and beam scanning module. The system has two photomultiplier tube detectors for parallel acquisition of two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) signals. A customized metallic ring taped on the subject's skin attaches magnetically to the objective holder in the articulated arm, minimizing motion artifacts. The images

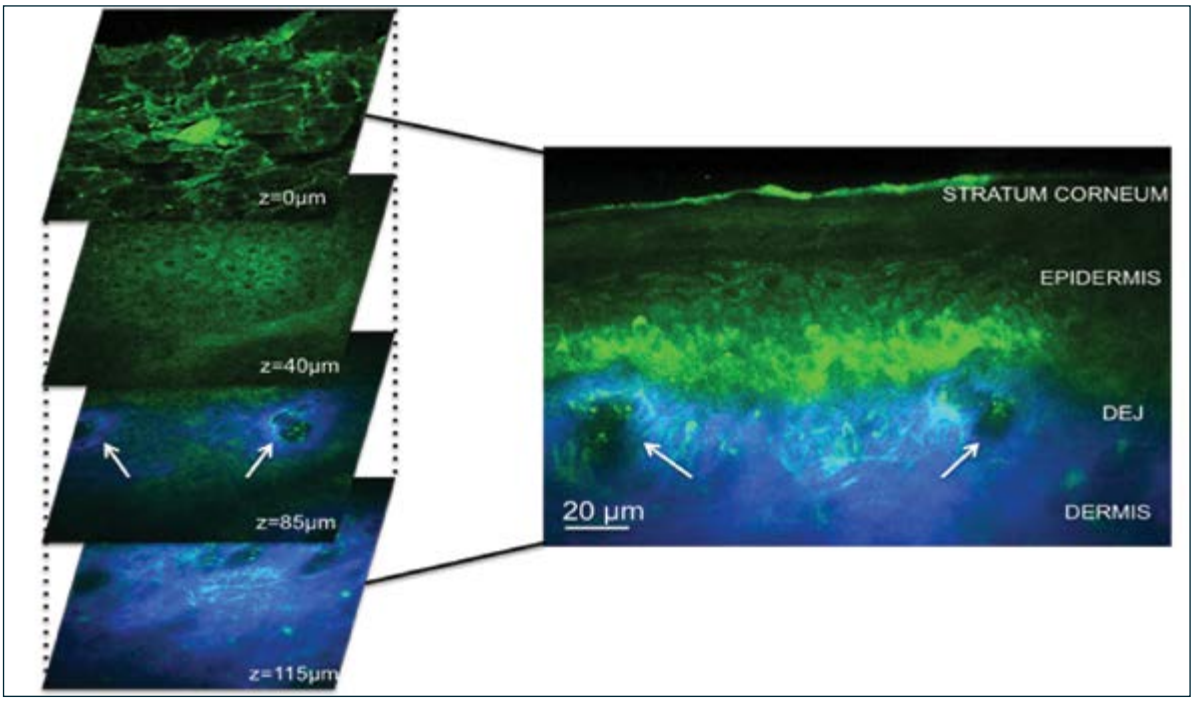

Figure 2. In vivo MPM imaging of normal skin (volar forearm). (Left) MPM horizontal sections (XY scans) at different depths showing images of the stratum corneum (z=0μm), keratinocytes normally distributed in the stratum spinosum (z=40μm), the basal cells (green) surrounding dermal papilla (blue) (z=85μm), collagen (blue) and elastin (green) in the dermis (z=115μm). (Right) Cross-sectional view (XZ scan) corresponding to a vertical plane through the horizontal sections on the left. The image shows the well-delineated layers of the stratum corneum, epidermis, dermal-epidermal junction (DEJ) and the superficial dermis. Arrows point to blood vessels. The dark circular shape inside each keratinocyte corresponds to the nucleus of the cell

acquired by MPTflex and DermaInspect have a lateral spatial resolution of ~0.5 μm and an axial resolution < 2 μm. The imaging depth in skin is about 200 μm, depending on the skin or lesion types imaged. The area imaged (field of view) is 250x250 μm^2. The field of view can be increased to a few mm^2 by implementing a mosaic "tiling" feature (acquisition of adjacent fields of view).

In the last years, both MPTflex and its former version DermaInspect have been employed in a broad range of clinical applications spanning from skin cancer detection and diagnosis [7-9], to cosmetic treatments [10], skin aging [11-14] and characterizing and understanding keratinocyte metabolism [15].

Applications of MPM-based clinical tomographs in dermatology

In vivo MPM imaging of normal skin

Commonly, MPM imaging is used in en-face (horizontal sections) mode, but cross-sectional (vertical) sections can also be acquired in real-time. Figure 2 shows a representative example of MPM images corresponding to normal skin and acquired with an MPTflex system. The en-face images were acquired as a z-stack of horizontal images every 5μm. En-face images corresponding to different skin layers were selected and shown in Fig. 1. The stratum corneum is visualized through the TPEF fluorescence from keratin. It appears as a thin bright layer on the top surface of the skin in the cross-sectional image and as an acellular bright fluorescent layer in the corresponding en-face image. The epidermis shows normally distributed keratinocytes imaged by the TPEF fluorescence from NADH/FAD, keratin and melanin (in the case of pigmented skin). The dermal-epidermal junction (DEJ) is clearly delineated in the cross-sectional view as it separates the basal cell layer from the dermis. Pigmented keratinocytes in the basal layer appear as bright fluorescent cells along the DEJ due to their melanin content. In the en-face images of the DEJ the basal cells are imaged as surrounding the tips of the dermal papillae. Dermal papillae and the dermis are visualized through the SHG signal from collagen and TPEF signal from elastin fibers. Occasionally, blood vessels and capillaries are imaged in the dermis as shown in Fig. 2.

In vivo MPM imaging of melanoma

Melanoma is the most severe form of skin cancer. It arises from melanocytes, the cells responsible for producing pigment. There are several subtypes of melanoma. Their features have differences and similarities, but generally the following are suggestive of malignancy: presence of melanocytes within the upper portion of the epidermis singly or in groups (Pagetoid spread); irregular junctional activity (atypical melanocytes, archi-

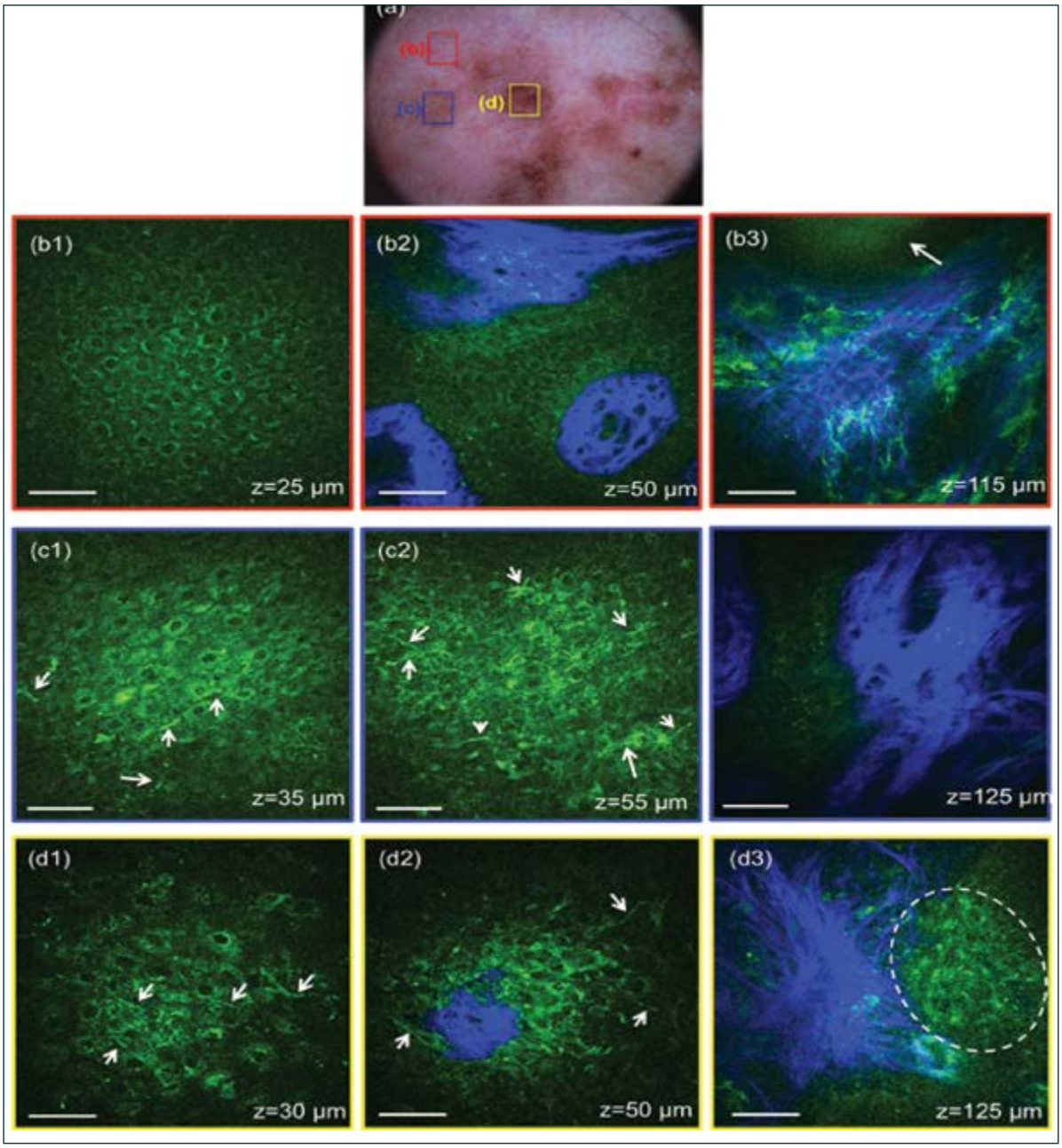

Figure 3. MPM images of a micro-invasive melanoma lesion on a patient's forehead (a) Clinical image (DermLite FOTO, Dermlite Inc.). The markers represent the locations where the images shown in (b1-b3), (c1-c3) and (d1-d3) were acquired. (b1-b3) MPM images corresponding to normal skin adjacent to lesion (red square in a) showing normal distribution of keratinocytes (b1), the basal cells (green) surrounding dermal papilla (blue) at 50 µm depth (b2) and collagen (blue) and elastin (green) fibers surrounding a hair follicle (arrow) in the dermis at a depth of 115 µm (b3). (c1-c2) MPM images of the epidermal layers corresponding to different depths of the lesion area indicated by the blue square in (a). The images show ascending melanocytes (white arrows) (c1-c2) and no obvious features of malignancy in the papillary dermis (c3). (d1-d3) MPM images of the lesion area indicated by the yellow square in (a). The images show ascending melanocytes (white arrows) in upper epidermal layers (d1), proliferation of melanocytes (white arrows) at DEJ (d2) and of pigmented cells with different shapes and enlarged nuclei (cellular atypia) in the dermis at a depth of 125 µm. Scale bar is 40 µm

tectural disorder); and invasion of tumor cells into the dermis [7, 16, 17]. These features have been identified in melanoma lesions in a study published in 2009, where a DermaInspect MPM tomograph was used to establish sensitivity and specificity criteria for melanoma diagnosis [9]. The assessment by different observers of these features led to overall sensitivity and specificity values for melanoma diagnosis of 75% and 80%, respectively. In that study as well as in most of the RCM studies on *in*

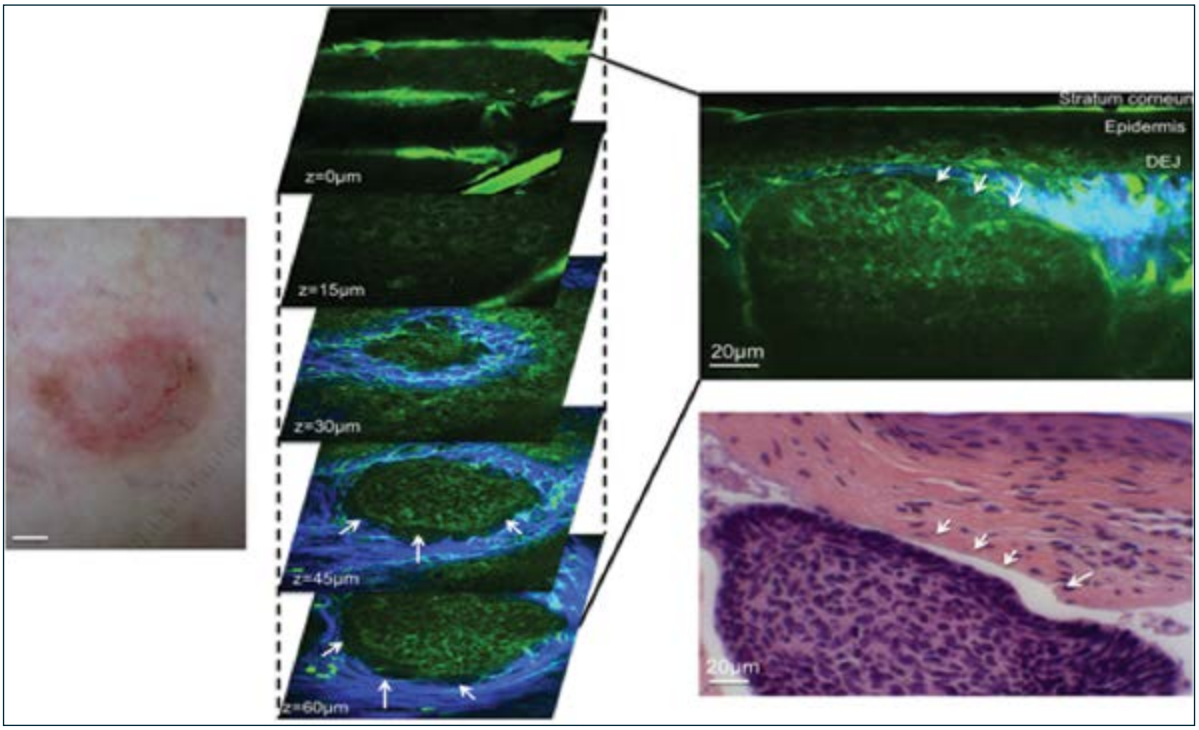

Figure 4. MPM images of a superficial BCC lesion on the shoulder of a patient (a) Clinical image (DermLite FOTO, Dermlite Inc.). Scale bar is 2 mm. (b1-b5) MPM en-face images (XY scans) of the stratum corneum at z=0μm (b1), keratinocytes in the stratum spinosum at z=15μm (b2), a nest of basaloid cells (green) surrounded by collagen (blue) and elastin fibers (green) imaged at different depths: z=30μm, 45μm, 60μm (b3-b5). (c) Cross-sectional view (XZ scan) corresponding to a vertical plane through the same interrogating volume shown on the left. (d) H&E stained histologic section of the lesion. Both the MPM and the histologic images show a mucinous stroma adjacent to the tumor mass (arrows). Reproduced with permission from Ref. [8]

vivo imaging of melanoma, particular features of atypical/dysplastic nevi are hardly discussed. In a more recent pilot study, our group employed an MPM-based MPTflex tomograph to identify characteristic features of 15 melanocytic nevi at three different stages: common nevi without dysplastic changes, dysplastic nevi with structural and architectural atypia, and melanoma (5 in each group) (7). We proposed and developed a quantitative approach by translating the qualitative features used by dermatopathologists in histopathology into quantitative parameters that can be uniquely extracted from 3D *in vivo* MPM images. We defined a numerical "multiphoton melanoma index (MMI)" based on 3D *in vivo* image analysis that scores signals derived from TPEF, SHG, and melanocyte morphology features on a continuous 9-point scale. Indices corresponding to common nevi (0–1), dysplastic nevi [1-4] and melanoma [5-8] were significantly different ($p<0.05$), suggesting the potential of the method to distinguish between melanocytic nevi *in vivo*[7]. Nevertheless, a more comprehensive study of a larger number of patients is necessary in order to validate the proposed scoring algorithm and evaluate how well MPM technology can distinguish dysplastic nevi from common nevi and melanoma. Figure 3 illustrates representative MPM images of a micro-invasive melanoma lesion along with the MPM images corresponding to normal skin adjacent to lesion. The lesion was imaged prior to biopsy. The intra-epidermal proliferation of melanocytes and the upward intraepithelial spread shown in the MPM images of Figure 3 were confirmed by histopathology.

In vivo MPM imaging of basal cell carcinoma (BCC)

BCC is a form of skin cancer that originates from the basal cells of the epidermis and associated follicular structures. Non-melanoma skin lesions, such as BCC have been imaged *in vivo* by MPM only recently in two pilot research clinical studies [8, 18]. In a first pilot study evaluating the *in vivo* MPM imaging of BCC lesions, the identified features were compared to the ones provided by RCM imaging [18]. Besides the two main features: elongated, polarized nuclei and tumor nests showing peripheral palisading identified by both techniques, MPM had the ability to evaluate changes in the nucleus/cytoplasm ratio and in cell density across the epidermal layers of BCC lesions. In a more recent study, the MPTflex tomograph was used in order to evaluate if histopathologic criteria can be identified in MPM images [8]. In this study, the use of MPTflex allowed imaging lesions on different parts

of the body rather than being limited to lesions on the extremities, as in the previous work, in which the DermaInspect was employed for MPM imaging [18]. This work demonstrated the ability of MPM to identify the main histopathologic criterion for BCC diagnosis in all the lesions imaged: tumor nests of basaloid cells at the DEJ and/or in the dermis that often showed palisading. The MPM ability to resolve the cellular structure inside the tumor nests is critical in distinguishing BCC from other types of neoplasms that involve presence of nests in the dermis. Figure 4 depicts representative MPM images of a BCC lesion imaged by MPM.

Other applications of MPM in dermatology

Aside from melanoma and non-melanoma skin cancers, MPM clinical tomographs have also been employed to imaging other skin conditions such as actinic keratosis [19, 20], atopic dermatitis [21] and pemphigus vulgaris[20]. The morphological features identified in actinic keratosis lesions were associated with wide inter-cellular spaces between keratinocytes, an increase in the ratio of nuclear to cellular size of keratinocytes and an increase in epidermal thickness compared to adjacent normal skin [19]. In atopic dermatitis lesions, changes in the cellular metabolism of the lesions have been *assessed in vivo* through protein-bound and free NADH fluorescence lifetime measurements [21].

Monitoring the effects of skin treatments, a key area of focus in cosmetic industry, is another field of interest for MPM imaging. In a study published in 2010, Bazin *et. al* used a DermaInspect MPM tomograph to evaluate the effects of a cosmetic product (topical cream) on the collagen and elastin fibers of the forearms of 24 subjects [10]. The ratios between the signals from collagen and elastin were measured at different depths in the dermis for each subject. After 12 weeks of treatment, an increase in the collagen/elastin ratio was measured for the cosmetic product containing active components (soy and jasmine) known to increase collagen synthesis. The treatment with a placebo topical cream (no active ingredients) resulted in no significant variation in the collagen/elastin ratio over the same treatment period of time.

In order to evaluate cosmetic treatments targeting skin anti-aging, approaches to quantify skin aging non-invasively have been investigated by using MPM imaging. Lin *et al.* proposed a dermis index that estimates the skin age by the ratio of the SHG signal from collagen and the TPEF signal from elastin [22]. The correlation of this index with age was demonstrated by *in vivo* MPM measurements of sites on subjects' forearms and faces using a DermaInspect tomograph [11, 13]. Kaatz performed depth-resolved measurements of the proposed dermis index and its correlation with age [12]. MPM imaging has also been used by Koehler *et al.* to evaluate in vivo and non-invasively dermal elastosis, a characteristic of skin photoaging [14].

In addition to skin conditions diagnosis and treatments, *in vivo* MPM imaging has also been applied to monitoring of the supply of oxygen to the mitochondria within epidermal cells [15]. In this study, the ischemia-induced oxygen deprivation was associated with a strong increase in NADH fluorescence of keratinocytes in layers close to the stratum basale, whereas keratinocytes from epidermal layers closer to the skin surface were not affected. This outcome supports the hypothesis that the vascular contribution to the basal layer oxygen supply is significant and these cells engage in oxidative metabolism. Keratinocytes in the more superficial epidermal layers are either supplied by atmospheric oxygen or are functionally anaerobic [15]. Real-time, in vivo noninvasive monitoring of keratinocytes metabolism is important for clinical purposes and for understanding of cellular physiology and cytology.

Discussion

The recent introduction of MPM for clinical studies in dermatology opened a new way to investigate human skin by imaging the epidermis and superficial dermis *in vivo* and non-invasively. Recent clinical studies have shown that MPM can be a potential tool for non-invasive imaging of different skin conditions including skin cancer, for monitoring cellular metabolism and the effects of skin treatments. While these are promising results indicating that real-time non-invasive "optical biopsies" can be performed at the bedside, translation of this technology into clinical practice seems to advance rather slowly due to several technical and practical challenges, which we discuss below.

In this early stage of MPM technology clinical translation, limited field of view (about 250x250 μm^2) and penetration depth (about 200-300 μm) are the main *technical challenges*. The field of view can be increased by implementing a mosaic "tiling" feature (acquisition of adjacent fields of view) or by re-design of the optical components. This technical limitation is being addressed and implementation of tiling has been initiated in a newly developed MPTflex model. Penetration depth can be improved by employing dispersion compensation to decrease the laser pulse duration, but the gain would be limited. Generally, this technology is intended as an aid for dermatologists to improve their clinical diagnosis of early stage skin diseases when the uncertainty of their decision is likely to be higher than in the case of advanced disease.

Practical challenges are related to the need of establishing a correlation between the MPM and the histological images. Most importantly, the MPM provides high-resolution images that represent horizontal optical sections within a tissue. In histopathology, histological cross-sections of the biopsied tissue are used for diagnosis. Secondly, while the MPM provides images of the cellular structure and the extra-cellular matrix of the real tissue, the images of the histological sections contain artifacts due to tissue processing and staining. A relevant example is the different appearance of melanocytes, in the MPM images as dendritic cells, and in the hematoxylin and eosin (H&E) stained histo-

logical sections, as highly pigmented cells sitting in a lacuna due to a marked fixation retraction artifact [23]. Thirdly, the different color of the MPM and histology images might also be a barrier toward clinical acceptance of MPM. In MPM, images are usually color-coded using the primary colors: red, green or blue to represent the TPEF and the SHG signals. Histology is based on stains and images that have real color. For instance, the H&E histology is based on two stains (hematoxylin for nuclei, eosin for cellular cytoplasm and extra-cellular matrix of dermis) and the images appear purple and pink. This potential barrier can be addressed by "digitally staining" the MPM images to mimic the histology appearance [24]. Generally, overcoming these practical challenges related to translation of MPM to clinical practice requires training on both sides, the microscopy specialists and the dermatopathologists, in order to generate a common language that eases communication.

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