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ISBN
9780819493576

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Publication Date
2013-02-22

DOI
10.1117/12.2004697

Clinical studies of pigmented lesions in human skin by using a multiphoton tomograph

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ABSTRACT

I*n vivo* imaging of pigmented lesions in human skin was performed with a clinical multiphoton microscopy (MPM)-based tomograph (MPTflex, JenLab, Germany). Two-photon excited fluorescence was used for visualizing endogenous fluorophores such as NADH/FAD, keratin, melanin in the epidermal cells and elastin fibers in the dermis. Collagen fibers were imaged by second harmonic generation. Our study involved *in vivo* imaging of benign melanocytic nevi, atypical nevi and melanoma. The goal of this preliminary study was to identify *in vivo* the characteristic features and their frequency in pigmented lesions at different stages (benign, atypical and malignant) and to evaluate the ability of *in vivo* MPM to distinguish atypical nevi from melanoma. Comparison with histopathology was performed for the biopsied lesions. Benign melanocytic nevi were characterized by the presence of nevus cell nests at the epidermal-dermal junction. In atypical nevi, features such as lentiginous hyperplasia, acanthosis and architectural disorder were imaged. Cytological atypia was present in all the melanoma lesions imaged, showing the strongest correlation with malignancy. The MPM images demonstrated very good correlation with corresponding histological images, suggesting that MPM could be a promising tool for *in vivo* non-invasive pigmented lesion diagnosis, particularly distinguishing atypical nevi from melanoma.

Keywords: multiphoton microscopy, melanoma, clinical tomograph, MPTflex

1. INTRODUCTION

The current standard for melanoma detection is based on clinical visual assessment of the lesions (ABCDE rule) [1], biopsy and histopathology. The ABCD approach is effective in distinguishing well between the two extreme stages of melanocytic lesions: common nevi and melanoma. The diagnostic accuracy of the intermediate stages of melanocytic nevi is further improved by dermatopathology through microscopic evaluation of the excised tissue. Although considered a gold standard for diagnosing melanocytic nevi, this routine diagnostic method is affected by limitations in discriminating early melanoma from atypical nevi for a large number of lesions that fall into the borderline area. This creates the problem of false-negative diagnosis that could delay diagnosis and treatment, and of false-positive diagnosis, which could lead to unnecessary biopsies and treatments.

Recently, non-invasive optical imaging technologies based on laser-scanning microscopy have emerged as promising tools for real time, *in situ* imaging of skin lesions with the potential, if clinically accepted, to reduce the amount of excision and specimen processing as in conventional histopathology. [2-4]

Among these, multiphoton microscopy (MPM) distinguishes itself as a laser-scanning microscopy technique that relies on nonlinear light-matter interactions such as two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) to achieve sub-micron resolution 3D images of tissues, at fast acquisition rates. Importantly, these contrast mechanisms produce images of endogenous biomolecules in the tissue, without using specific fluorescent labels. In MPM, the main sources of fluorescence are reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinuc

leotide (FAD), keratin, melanin and elastin fibers, while SHG is used to visualize the collagen fibers in the dermis.

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Multiphoton Microscopy in the Biomedical Sciences XIII, edited by Ammasi Periasamy, Karsten König, Peter T. C. So, Proc. of SPIE Vol. 8588, 858812 · © 2013 SPIE · CCC code: 1605-7422/13/$18 · doi: 10.1117/12.2004697

MPM has been used to image *in vivo* melanoma lesions in a study that established several sensitivity and selectivity criteria for melanoma diagnosis [4]. Our study intends to complement the previous work by using the MPM imaging to identify *in vivo* the characteristic features and their frequency in pigmented lesions at different stages (common acquired or congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; melanoma) and to evaluate the ability of *in vivo* MPM to distinguish atypical nevi from melanoma.

This is important in order to improve the diagnostic accuracy of melanocytic lesions in all stages from benign nevi to metastatic melanoma, which can potentially lead to reducing unnecessary biopsies and unnecessary treatment of patients.

2. MATERIALS AND METHODS

2.1 MPTflex clinical tomograph

The laser-scanning based clinical multiphoton tomograph as shown in Figure 1, consists of a compact, turn-key femtosecond laser (MaiTai Ti:Sappire oscillator, sub-100 fs, 80 MHz, tunable 690-1020 nm; Spectra Physics, Mountain View, CA), an articulated arm with near-infrared optics and beam scanning module. The system has two photomultiplier tube (PMT) detectors employed for parallel acquisition of TPEF and 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 excitation wavelength used was 790 nm unless otherwise specified. The TPEF signal was detected over the spectral range of 410nm-650nm while the SHG signal was detected over a narrow spectral bandwidth 385nm-405nm through emission filters placed in the TPEF and SHG detection channels, respectively.

Figure 1. MPTflex - laser-scanning multiphoton microscopy - based clinical tomograph

2.2 Study design

In this study, melanocytic nevi at three different stages were imaged: common acquired or congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; melanoma.

We performed the comparison of the MPM images with histopathology images for all the biopsied lesions. Knowing and understanding the histological features characteristic to melanocytic nevi in different stages is important for two reasons: 1) it helps identify the characteristic microscopic features during the MPM imaging of the horizontal sections at different depths in the lesion and 2) it helps identify and evaluate the traditional histopathology criteria and their applicability to MPM images. Figure 2 shows the representative H&E histology images corresponding to different stages of melanocytic nevi. [5]

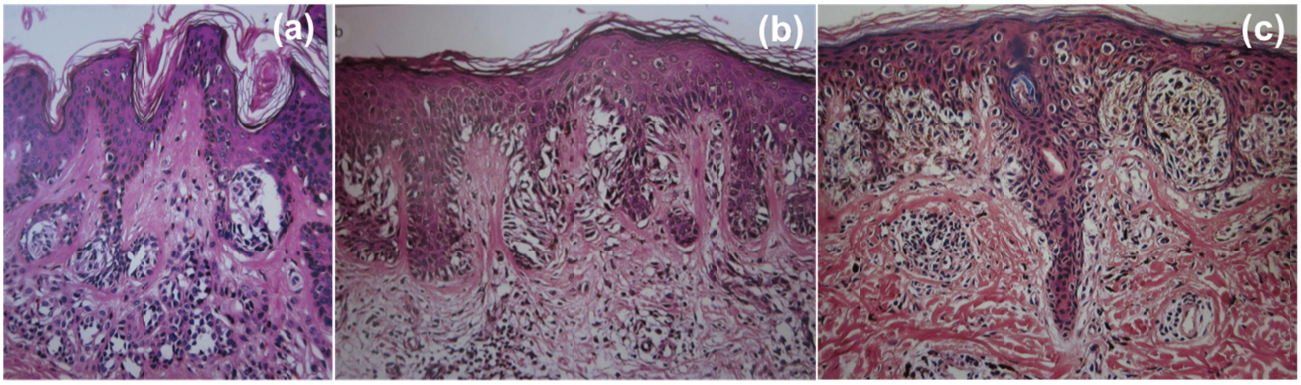

Figure 2. H&E representative histology images corresponding to (a) compound melanocytic nevus showing nests of melanocytes located at the tips of the rete ridges and in the dermis, (b) atypical nevus showing lentiginous hyperplasia and the abnormal location of the junctional nests along the sides of the rete and over the tips of the dermal papillae, (c) in situ melanoma showing presence of tumor intraepidermal cells (pagetoid spread)

MPM measurements were performed on the lesional site as wells as on an area adjacent to the lesion area (healthy skin). Optical sections of about 200x200 μm^2 at different depths ranging from 0 to 200 µm (5 µm steps) were obtained. The time required for each optical section was 6 s. As the optical section is limited to a small scan field, the overall investigation of the lesion required several image stacks of different skin sites. Total time for imaging a single lesion varied from 15 to 30 min.

2.3 Subjects

In this preliminary study, 10 patients were imaged: 3 patients with melanocytic nevi (common acquired or congenital nevi without dysplastic changes), 4 patients with atypical nevi and 3 patients with melanoma. 6 of the 10 patients imaged underwent biopsy procedure (3 atypical nevi and 3 melanoma were biopsied). All in vivo measurements were conducted according to an approved institutional protocol, and with informed consent by all participants.

3. RESULTS AND DISCUSSIONS

We performed MPM imaging of pigmented lesions in three stages (common acquired or congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; melanoma) in an effort to identify the characteristic features and their frequency in pigmented lesions at different stages and to evaluate the ability of in vivo MPM to distinguish atypical nevi from melanoma.

3.1 Melanocytic nevi (without dysplastic changes)

We imaged 3 patients with melanocytic nevi (common acquired or congenital nevi without dysplastic changes), all three diagnosed clinically as compound nevi. The MPM features of melanocytic nevi were characterized by normal morphology of keratinocytes of the epidermal layers, nests of nevus cells surrounded by collagen fibers at the epidermal-dermal junction (EDJ) and elongated rete ridges compared to normal skin. Melanocytic nevi lesions were not biopsied, but the features identified by the MPM imaging were in good correlation with features generally identified by histopathology in compound nevi (Figure 2a).

Figure 3 shows MPM images of a compound nevus corresponding to different depths along the EDJ.

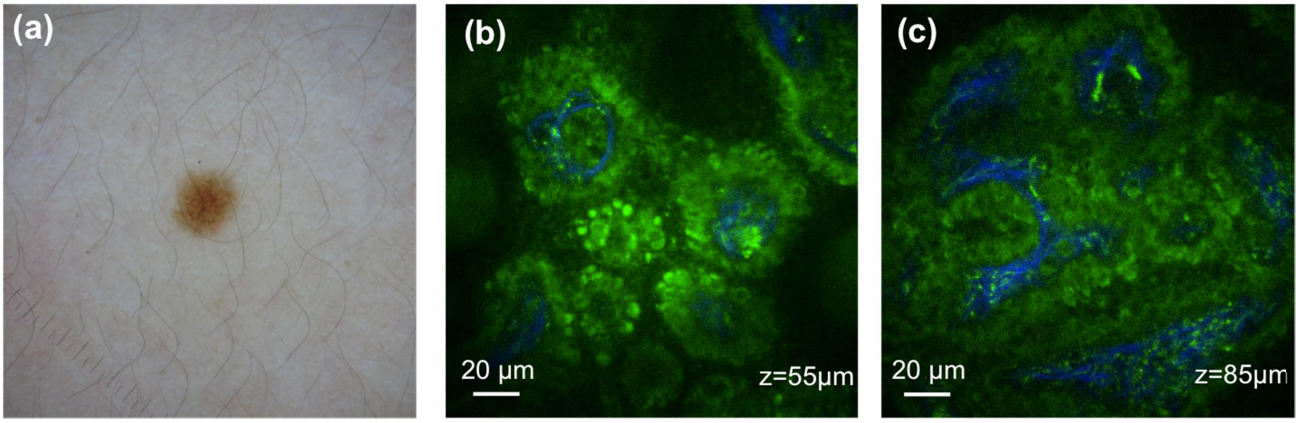

Figure 3. Clinical image of a compound nevus (a) MPM images of the nevus showing nests of nevus cells (green) surrounded by collagen fibers (blue) at a depth of 55 µm (b) and 85 µm (c) along EDJ

3.2 Atypical nevi

Four patients with atypical nevi were imaged in this preliminary study. MPM features such as architectural disorder of keratinocytes in the stratum spinosum was observed in one lesion, while lentiginous hyperplasia (nevus cells with dense distribution along the basal layer) was observed in three of the four lesions imaged and acanthosis (thickening of the epidermal layer) was observed in one lesion. Representative images of one of the atypical nevi along with corresponding histology are shown in Figure 4. The diagnosis based on histopathology corresponding to this lesion was: compound dysplastic nevus with mild to moderate atypia. The MPM features were characterized by architectural disorder of keratinocytes, lentiginous hyperplasia and acanthosis.

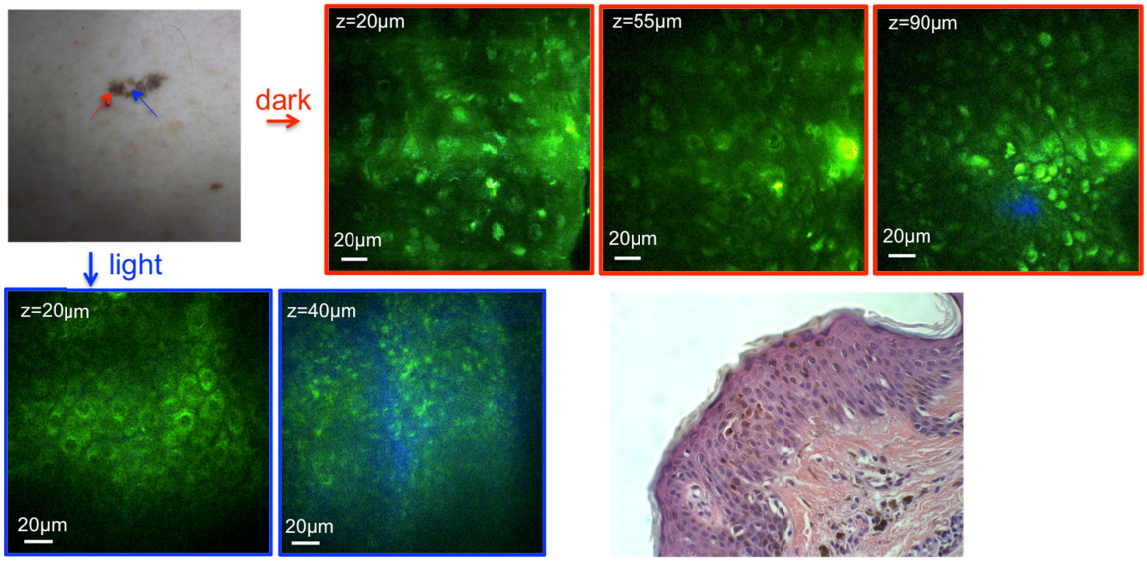

Figure 4. *Top row:* (Left) Clinical image of an atypical nevus. Red arrow points to the dark area corresponding to MPM images on the right. Blue arrow points to the light area corresponding to MPM images on the bottom row. (Right) MPM images at different depths corresponding to the dark area of the lesion showing architectural disorder of keratinocytes, lentiginous hyperplasia and acanthosis. *Bottom row:* (Left) MPM images of the epidermis and EDJ corresponding to the light area of the lesion showing normal distribution of keratinocytes. (Right) Histopathology image of the lesion showing acanthosis and lentiginous hyperplasia

3.3 Melanoma

We imaged 3 patients who were diagnosed with melanoma based on histopathology. The lesions were diagnosed as follows: melanoma in situ (2) and melanoma-lentigo type (1). The MPM features of the melanoma lesions were characterized by cytological atypia, migration of melanocytic dendrites into the spinosum layer of the epidermis, architectural disorder and erosion of the EDJ (in the melanoma-lentigo type lesion). These features correlate well with the features of the corresponding histological images.

MPM images corresponding to one of the melanoma in situ lesions along with the histology image are shown in Figure 5. The MPM features were characterized by pagetoid cells (large pleomorphic cells) and melanocytic dendrites imaged in the upper layers of the epidermis.

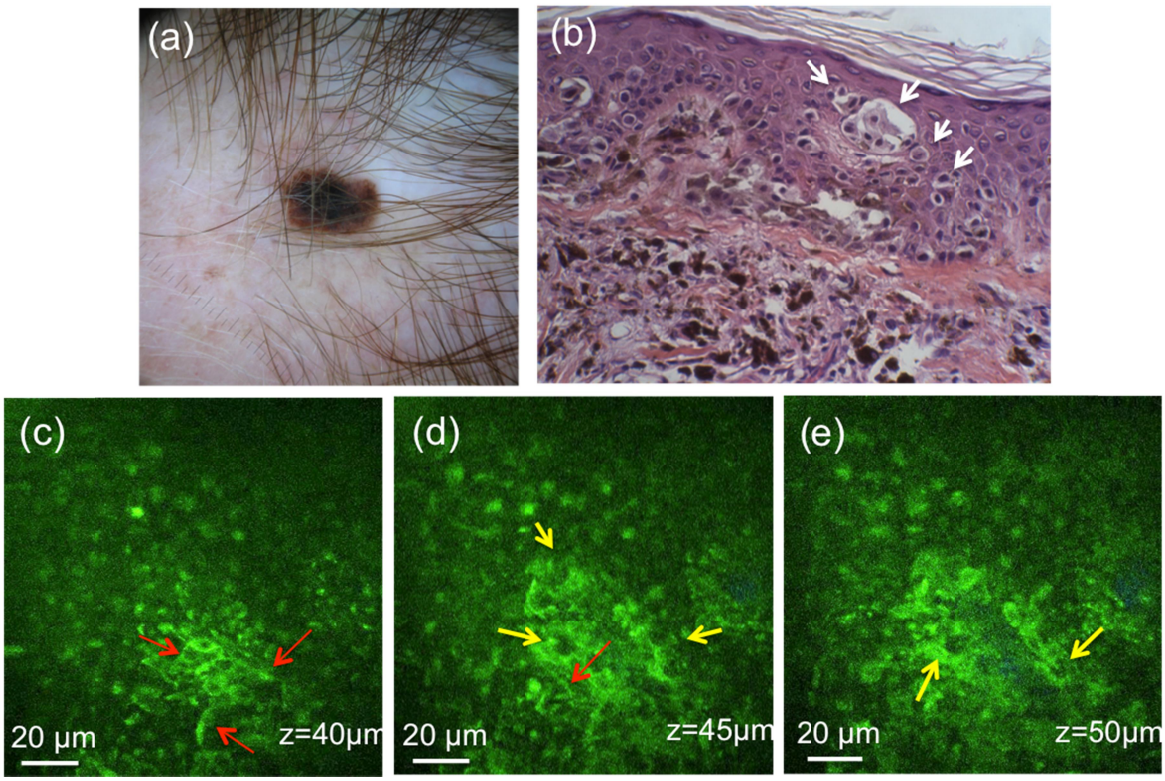

Figure 5. Clinical image of a melanoma lesion (a) Histology image of the lesion (b) White arrows point to pagetoid cells and melanocytes advanced in the upper layers of the epidermis. MPM images of the lesion at different depths: 40 µm (c), 45 µm (d) and 50 µm (e). Red arrows point to melanocytic dendrites in the spinosum layer of the epidermis. Yellow arrows point to pagetoid cells in the spinosum layer of the epidermis.

Table 1 summarizes the MPM features and their frequencies observed in the epidermis, epidermal-dermal junction and papillary dermis in normal melanocytic nevi, atypical nevi and melanoma. Cytological atypia appears to show the strongest correlation with malignancy. Melanocytic dendrites advanced in the stratum spinosum were present in some of the atypical nevi and some of the melanoma lesions. In these cases, the architectural disorder influenced the overall grade.

Table 1. MPM features and their frequencies observed at the epidermal layers, epidermal-dermal junction and papillary dermis in normal melanocytic nevi, atypical nevi and melanoma

Lesion/MPM features	**Epidermis Stratum granulosum/spinosum**	**Basal layer/Epidermal-dermal junction**	**Papillary dermis**
Melanocytic nevi (3)	normal	nests of nevus cells surrounded by collagen (3)	normal
Atypical nevi (4)	- presence of melanocytic dendrites in stratum spinosum (2) - architectural disorder (1) - acanthosis (1)	lentiginous hyperplasia (3)	normal
Melanoma (3)	- presence of melanocytic dendrites in stratum spinosum (2) - presence of pagetoid cells (1)	-cytological atypia (3) - presence of pagetoid cells (1) -erosion of the junction (1) -architectural disorder (3)	invasion of melanocytes (1)

These preliminary data suggest that multiphoton microscopy could be a promising tool for distinguishing melanoma from atypical nevi *in vivo* and non invasively. Nevertheless, images from a larger number of patients need to be acquired and statistics performed in order to evaluate sensitivity and specificity for MPM diagnosing of melanocytic nevi, atypical nevi and melanoma.

4. CONCLUSION

A multiphoton clinical tomograph was employed to image *in vivo* pigmented lesions of human skin at three different stages: (common acquired or congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; melanoma). The goal of this preliminary study was to identify the characteristic features and their frequency in pigmented lesions at the three different stages and to evaluate the ability of in vivo MPM to distinguish atypical nevi from melanoma. The good correlation between the features identified by MPM and those corresponding to histological images, suggests that MPM could be a promising tool for *in vivo* non-invasive pigmented lesion diagnosis, particularly distinguishing atypical nevi from melanoma. Nevertheless, images from a larger number of patients need to be acquired in order to evaluate major and minor diagnosis criteria and determine sensitivity and specificity criteria for MPM diagnosing of melanocytic nevi, atypical nevi and melanoma.

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ACKNOWLEDGEMENTS

Support for this work was provided by the National Institutes of Health (NIH) Laser Microbeam and Medical Program (LAMMP, P41-EB015890) and the Chao Family Comprehensive Cancer Center (P30CA62203). Support from the Arnold and Mabel Beckman Foundation and Air Force Research Laboratory Agreement No. FA9550-04-1-0101 is acknowledged. Karsten König is cofounder of JenLab GmbH.