ABSTRACT

Multiphoton microscopy is a recent non-invasive skin imaging technique that allows the human skin three-dimensional structure to be characterized in vivo with sub-µm resolution. This technique, in combination with specific 3D image processing tools, allows an automatic segmentation of the different skin layers and an extraction of several quantitative parameters characterizing skin in terms of morphology, density and organization. In this paper, we propose a new specific 3D melanin detection and quantification method based on multiphoton microscopy and fluorescence lifetime imaging, compatible with in vivo human skin investigations. We applied this specific melanin analysis method in two clinical trials: i) 15 young (18-25y) and 15 aged (70-75y) human female volunteers focusing on the ventral and dorsal sides (unexposed vs. exposed) of their forearm, and ii) 45 female volunteers (18-55y) with different skin phototypes (ITA values of their ventral forearm ranging from grade I to V). Our specific 3D melanin quantification method allowed us to bring out age related melanin changes between the ventral and dorsal sides of the forearm and discriminate the different skin phototypes. This innovative approach could have large applications in both the dermatological and cosmetic fields, since easily applied to the characterization of pigmentation disorders or to the assessment of compounds able to modulate skin melanin content.

Keywords: skin, melanin, quantification, in vivo, multiphoton microscopy, 3D image processing, second harmonic generation, two-photon excited fluorescence.
We recently demonstrated that multiphoton microscopy, in association with specific 3D image processing tools [1,3], offers the possibility to automatically segment the different skin layers and extract several quantitative parameters characterizing skin in terms of morphology, density and organization. For example, the different skin layers thickness can be quantified, the shape of the dermal-epidermal junction (DEJ) or the 3D density of elastic and collagen fibers. Melanin quantification can also be performed in the basal layers of epidermis, where melanin is often highly concentrated and shows 2PEF signal intensities stronger than that of other endogenous fluorophores[1]. But for melanin quantification, methods based upon fluorescence intensity levels are not always satisfactory. First, the signal intensity may be disrupted by other fluorophores of a strong fluorescence intensity (e.g. keratin in the stratum corneum), and second, it does not take into account pixels with low melanin concentration of a fluorescence intensity comparable to that of other endogenous fluorophores.

A more specific method takes into account the melanin fluorescence lifetime (FLIM - Fluorescence-Lifetime Imaging Microscopy). However, classical FLIM imaging is too long to obtain in vivo 3D data, and in practice is limited to selected 2D slices, obtained at a depth chosen by the operator.

In this paper, we propose a new specific 3D melanin detection and quantification method based on multiphoton microscopy and fluorescence lifetime imaging [11], compatible with in vivo human skin investigations: “Fast-FLIM” melanin analysis.

MATERIALS AND METHODS

PATIENTS

Two clinical trials were performed with the multiphoton medical device DermaInspect™ whose experimental protocols were approved by the Saint Louis Hospital ethics committee, complying with the Declaration of Helsinki. These two studies involved i) 15 young (18-25y) and 15 aged (70-75y) human female volunteers focusing on the ventral and dorsal sides (unexposed vs. exposed) of their forearm, and ii) 45 female volunteers (18-55y) with Individual Typology Angle (ITA) [12] values of their ventral forearm ranging from grade I to V.

MULTIPHOTON IMAGING

Combined SHG/2PEF imaging was performed using the DermaInspect™ device (JenLab GmbH, Jena, Germany), a CE-marked medical imaging device integrating two Time-Correlated Single Photon-Counting (TCSPC) detectors for fluorescence lifetime imaging (FLIM) (SPC-830, Becker & Hickl, Berlin, Germany), as previously described [2]. SHG and 2PEF-FLIM signals were simultaneously excited by a femtosecond Titanium-Sapphire laser adjusted to 760 nm (MaiTai Spectra-Physics, Mountain View, CA, USA) and by use of a 40x, 1.3-NA oil immersion objective lens (Carl Zeiss, Jena, Germany). The skin was imaged through a cover glass placed at the skin surface and a small drop of water was used for the optical contact between the skin and the cover glass. The excitation power was exponentially increased from 12 mW at the surface of the skin up to 47 mW at a depth of 75 µm, and kept constant for imaging depths greater than 75 µm.

A multiphoton 3D (x, y, z) image of 130x130x162 µm³ volume corresponds to a stack of 70 en face images of 511x511 pixels (0.255 µm/pixel) acquired with 2.346 µm z-step. For each pixel, the multiphoton FLIM signals, acquired by the TCSPC detectors, were integrated using only four temporal channels with an integration time of 2 ns per channel. The total acquisition time was 7.4 s per image (511x511 pixel) and 9.4 min /3D image. For each volunteer, two 3D images were acquired per condition in two adjacent regions of the ventral and / or dorsal forearm side. Each 3D image starts and ends respectively at -10 µm above and 150 µm below the skin surface.

3D IMAGE PROCESSING

The 3D images were analyzed using automatic 3D image processing tools we recently developed [1,3,10]. Briefly, we automatically separate the different skin layers (stratum corneum, living
epidermis (LED), epidermis, dermis), characterize the 3D DEJ shape and extract quantitative parameters on the different skin constituents and layers.

Melanin quantification was performed using a specific melanin detection procedure associating multiphoton microscopy and fluorescence lifetime imaging [11]. Melanin density corresponds to the ratio of the number of voxels (“3D pixels”) occupied by melanin and the total number of voxels in the volume occupied by the living epidermis. This “Fast-FLIM” melanin analysis processing is based on reduced FLIM data analysis, i.e. 4 “long” time channels covering the whole decay curve being used instead of many “short” time channels.

Figure 1: (Left) Typical *in vivo* multiphoton images of normal forearm human skin acquired at different depths within the epidermis and (right) corresponding melanin mask obtained using our specific “Fast-FLIM” melanin analysis procedure. 2PEF signal (cyan hot color) reveals the endogenous fluorophores distribution inside the epidermis: (top) corneocytes in the stratum corneum; (middle) keratinocytes in the stratum granulosum and (bottom) keratinocytes close to the dermal-epidermal junction (basal layers of the epidermis). The melanized keratinocytes with high melanin 2PEF intensity signal are highlighted in white.
Using the 3D automatic segmentation method described above [3], we could extract quantitative parameters such as the global 3D melanin density in the epidermis. In addition, normalized layers inside the 3D-delimited epidermis were defined in depth and a specific algorithm led to the normalized profile of melanin as a function of normalized depth, from the stratum basale (dermal-epidermal junction) up to the stratum corneum (skin surface).

RESULTS AND DISCUSSION

Typical in vivo multiphoton images of normal forearm human skin are shown in Figure 1; more images and a detailed description can be found in reference [2]. The corresponding melanin mask obtained using our specific “Fast-FLIM” melanin analysis procedure show the skin pixels occupied by melanin: more melanin at the basal layers and decreased melanin density at the level of stratum granulosum and stratum corneum.

We first studied the skin pigmentation differences as a function of age for both ventral and dorsal forearm side. The results of the melanin differences are shown in Figure 2. As expected, the melanin density is more important on the dorsal side compared to the ventral side, because the ventral side is more sun protected and less pigmented. This conclusion is confirmed by statistical analysis and is in agreement with the histological findings (data not shown).

Secondly, we applied this specific melanin analysis tools to the study of melanin changes between the different skin phototypes (ITA values measured on the ventral forearm ranging from grade I to V). As illustrated on Figure 3, we are able to discriminate the different skin phototypes: as expected and in agreement with histology, we evidenced an increase in the global mean melanin density with ITA group. Moreover, we studied the melanin density normalized z-profile from the dermal-epidermal junction up to the stratum corneum (skin surface). Our results show an increased melanin density at the basal layers for ITA groups II to V, almost no differences at the level of stratum granulosum and a slight increase in melanin density at the level of stratum corneum for darker skin color.
CONCLUSION

We proposed a new specific 3D melanin detection and quantification method, based on “Fast-FLIM” analysis of multiphoton fluorescence lifetime data. We demonstrated that this non-invasive method is compatible with in vivo human skin investigations and allows evidencing the constitutive pigmentation differences and pigmentation changes occurring with aging and sun exposure.

This innovative approach could have large applications in both the dermatological and cosmetic fields, since easily applied to the characterization of pigmentation disorders or to the assessment of compounds able to modulate skin melanin content. More generally, the association of multiphoton microscopy with specific image processing provides a powerful tool for 3D skin quantification which is of paramount importance for a non-invasive evaluation and quantification over time of dermatological treatments and cosmetic products effects.

REFERENCES


