**In vivo multiphoton multiparametric 3D quantification of human skin aging on forearm and face**

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**ABSTRACT**

Quantifying skin aging changes and characterizing its 3D structure and function in a non-invasive way is still a challenging area of research. In vivo multiphoton imaging offers means to assess skin constituents in 3D, however prior aging studies mostly focused on 2D analyses of dermal fibers through their signals’ intensities or densities.

We designed and implemented multiphoton multiparametric 3D quantification tools for in vivo human skin characterization. Despite the limited field of view of the technic, investigation of 2 regions of interest (ROIs) per zone per volunteer were found to be a good compromise in assessing 3D skin constituents in both epidermis and superficial dermis.

Skin aging was characterized on different UV exposed areas - ventral and dorsal forearms, face. Epidermal morphological changes occur late and were only objectified between extreme age groups. Melanin accumulated with age and chronic exposure on both forearms and appears earlier. Superficial dermal changes were mainly elastin density increase, also structurally modified from thin and straight fibers in young to dense and compact pattern in elder, and no obvious change in collagen density, both reflected by SHGto2PEF ratio and SAAID index decrease and ImbrN index increase. The second 20 µm-thickness normalized dermal sub-layer exhibited the highest aging differences. The 3D ImbrN index refines the share of photoaging in global aging on face and the 3D SAAID index on forearm.

Multiphoton multiparametric 3D skin quantification offers rich spatial information of interest in assessing normal human skin condition and its pathological, external environment or product induced changes.

**Keywords:** Multiphoton, Pseudo-FLIM, 3D quantification, epidermis, DEJ, melanin, dermis, elastin, fibrillar collagen, in vivo human skin aging, forearm, face

**1. INTRODUCTION**

Quantifying human skin aging especially on human face and more generally characterizing its 3D structure and function in a non-invasive way is still a challenging area of research, constantly evolving with the development / improvement of linear and non-linear optical imaging methods and image analysis tools. In vivo multiphoton imaging offers means to study skin aging and assess skin constituents in 3D, however prior skin aging studies mostly focused on 2D analyses of dermal fibers through their signals’ intensities or densities estimated at specific fixed depths around 110-160 µm bellow the skin surface. Combination parameters were introduced such as the SHG to autofluorescence aging index of dermis (SAAID) defined as the ratio of the difference to the sum of SHG and 2PEF pixels areas (2D-SAAID density-based index) or pixels intensities (2D-SAAID intensity-based index). Recently, by comparison with FLIM bi-exponential and phasor analyses, we have demonstrated how melanin can be detected in 3D by
multiphoton Pseudo-FLIM analysis (slope analysis of autofluorescence intensity decays from temporally binned data, compatible with 3D in vivo fast image acquisitions on human subjects)\textsuperscript{13, 14}. As the epidermal thickness may vary according to anatomical sites, skin ethnicity, aging or products application, we use both a global 3D epidermal melanin density parameter and a melanin z-epidermal distribution profile to compare skin regions with varying epidermal thickness.

In this work\textsuperscript{15}, we propose to take into account all the available 3D rich spatial information offered by multiphoton imaging and perform a global 3D assessment of in vivo human skin aging changes on ventral and dorsal forearms and face temple skin areas, after addressing the question of skin constituent’s variability and robustness of 3D multiphoton parameters for skin assessment.

2. RESULTS AND CONCLUSIONS

The full results of this work can be found in Pena et al. Scientific Reports 12, 14863 (2022)\textsuperscript{15}.
Figure 1: Global analysis process for in vivo 3D multiphoton images allowing 3D skin automatic layers segmentation and constituents quantification. a) Briefly, the first step of global 3D analysis of z-stacks of combined 2PEF-FLIM (4 time channels)/SHG images consists in identifying the epidermal and dermal layers (3D automatic segmentation) and quantifying their morphology (thickness, DEJ 3D-shape), the 3D global melanin density (in epidermis, SC, SG, LED and SB sublayers) and the 3D dermal density and organization of elastin and fibrillar collagens. b) A 3D high level epidermal segmentation is further performed for melanin z-epidermal distribution profile quantification. The 3D z-stacks of melanin masks calculated using the Pseudo-FLIM method\(^{13, 14}\) (purple color in the 2PEF-cyan hot/SHG-red images), the 3D automatic epidermis segmentation mask and its sublayers (3D high level epidermis segmentation; each color in the 3D reconstruction indicate a different epidermal sublayer) are jointly used in this process. By quantifying the 3D melanin density in 10-thickness normalized epidermal volume sublayers (from 1 - DEJ level to 10 - SC level), a 3D melanin z-epidermal distribution profile can be obtained. c) A 3D high level dermal segmentation is further performed for elastin and fibrillar collagens quantification. The imaged dermis is divided in 2 thickness normalized (10 or 20 µm thick) dermal volume sublayers that follow the DEJ shape (left and middle 3D reconstructions). A third layer is obtained, the remaining dermal sublayer. The imaged dermis can also be divided into a single 50 µm-thick dermal sublayer that follows the DEJ shape (right 3D reconstruction). Different dermal fibers parameters (intensities, densities, organization) can be calculated in the whole imaged dermal volume or within its sublayers. The data were acquired on the temple face skin area of a young volunteer. The 3D reconstructions were performed with Fiji, 3D Volume viewer. Reprinted from Ana-Maria Pena et al. “In vivo multiphoton multiparametric 3D quantification of human skin aging on forearm and face”, Scientific Reports 12, 14863 (2022), publisher Springer Nature, Copyright © 2022, The Author(s)\.\(^{15}\)

We performed 3 multiphoton clinical trials: Study I acquired with DermaInspect on ventral forearm (30-40 vs 55-65yo; “light” ITA vs “tan” ITA skin color groups); Study II acquired with DermaInspect on ventral and dorsal forearms (18-25 vs 70-75yo); Study III acquired with MPTflex on dorsal forearm and face temple area (18-25 vs 70-75yo).

Figure 1 summarizes the global analysis process implemented for in vivo 3D multiphoton images analysis and allowing 3D skin automatic layers segmentation and constituents quantification.

After checking the strong repeatability of the multiphoton imaging technique\(^{15}\), we first assessed the robustness of multiphoton quantification parameters that was lacking in the literature. Despite the relatively small investigated epidermal volume, we have previously shown that 3D morphological epidermal parameters (thickness and DEJ shape) as well as global 3D epidermal melanin density and its z-epidermal distribution\(^{14}\), enable studying various physiological conditions (constitutive and acquired pigmentation\(^{14}\), aging\(^{14}\), natural UV exposure\(^{16}\)) or treatment effect (topical retinoic acid and retinol\(^{16, 17}\) or corticosteroids\(^{18}\)).

Here, we further demonstrated that for in vivo multiphoton human skin studies, our experimental protocol (2 regions of interest (ROIs) per zone per volunteer) is robust compared to protocols involving 3 or 4 ROIs per volunteer and relevant in assessing in 3D normal human skin and its aging changes in terms of epidermal thickness, DEJ undulation, melanin density, elastin density, collagen density, SHGto2PEF densities ratio, SAAID density index and ImbrN – normalized imbrication (see full results in Pena et al.\(^{15}\)).

We then characterized skin aging on different UV exposed areas - ventral and dorsal forearms, face (data from 3 clinical trials, Figure 2). The three major facts of aging that are epidermal atrophy, the dermal-epidermal junction (DEJ) flattening and dermal elastosis can be non-invasively quantified and compared. Epidermal morphological changes occur late and were only objectified between extreme age groups.

Melanin accumulation in suprabasal layers with age and chronic exposure on ventral and dorsal forearms is less known and appears earlier (see full results in Pena et al.\(^{15}\)). Superficial dermal aging changes are mainly elastin density increase, with no obvious change in collagen density, reflected by SHGto2PEF ratio and SAAID index decrease and ImbrN index increase on all skin areas (Figure 3). Analysis of the z-dermal distribution of these parameters highlighted the 2nd 20 µm thickness normalized dermal sub-layer, that follows the DEJ shape, as exhibiting the highest aging differences (see full results in Pena et al.\(^{15}\)).

Moreover, the 3D ImbrN index allows refining the share of photoaging in global aging on face and the 3D SAAID index on forearm, which elastin or fibrillar collagens densities alone do not allow. Photooaging of the temple area evolves as a function of chronic exposure with a more pronounced increase in elastin density, also structurally modified from thin and straight elastic fibers in young volunteers to dense and compact pattern in older ones.
Figure 2: Multiphoton – Aging differences in 3D skin morphology of ventral and dorsal forearms and face temple area. 
a) 3D reconstructions of epidermal and dermal volumes of dorsal forearm and face temple area of a young and respectively old volunteers with epidermal thickness and DEJ undulation values close to the mean values of the study III groups. 
b1, b2, b3) Mean epidermal thickness; c1, c2, c3) Mean 3D DEJ undulation; d1, d2, d3) Mean stratum corneum thickness. The raw data are expressed as box plots with fences, mean and median (—). All ROIs values and their distribution (dots (♦) and histogram) are shown on the right side of the boxplot. Overlapping values are highlighted in black (★). In study I, the pink (♦) and green (♦) data points correspond to respectively women and men volunteers. Statistically significant p-values: *≤0.05; **≤0.01, ***≤0.001. The colored brackets indicate the ES – effect size (dark green - very strong [1.3 - Inf], light green - strong [0.8 - 1.3] and yellow - moderate [0.5 - 0.8]). Only p-values associated with moderate to very strong ES are shown. Reprinted from Ana-Maria Pena et al. “In vivo multiphoton multiparametric 3D quantification of human skin aging on forearm and face”, Scientific Reports 12, 14863 (2022), publisher Springer Nature, Copyright © 2022, The Author(s)15.
This work highlights the importance of using different 3D morphology, density and organization parameters to create a global picture of not only the dermal fibers but also of the epidermis morphology and its melanin pigment. This analysis could be completed by other measurements such as the size and number of cells or the fibers orientation estimated up to now only in specific 2D planes. Besides skin aging applications, we have previously shown the potential of 3D quantification tools to non-invasively characterize skin constitutive pigmentation and assess the effects of anti-aging compounds such as retinoic acid and retinol. More generally, multiphoton multiparametric 3D skin quantification offers rich spatial information of interest in assessing normal human skin condition and its pathological, external environment or product induced changes.

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