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Keywords:	two-photon excited fluorescence, fluorescence lifetime imaging, human skin, melanin, pigmentation, retinol, retinoic acid, aging

## ***In vivo* multiphoton imaging for non-invasive time course assessment of retinoids effects on human skin**

**Running title : Multiphoton imaging of retinoids effects**

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### **Conflict of interest:**

TB, SB, BN, AMP, ER, LS, ETB, SV are employees of L'Oréal Research and Innovation. One of the studied products (Retinol) is commercialized by L'Oréal Group. ED is one of the inventors of the patented 3D image processing tools used in this study. MB has no conflict of interest.

**Abbreviations:** ITA – Individual Typology Angle; 2PEF – two-photon excited fluorescence; SC – *stratum corneum*; LED – living epidermis; DEJ – dermal-epidermal junction; RO – Retinol; RA – Retinoic acid; ES – effect size;

## ABSTRACT

**Background:** *In vivo* multiphoton imaging and automatic 3D image processing tools provide quantitative information on human skin constituents. These multiphoton-based tools allowed evidencing retinoids epidermal effects in the occlusive patch test protocol developed for antiaging products screening. This study aimed at investigating their relevance for non-invasive, time course assessment of retinoids cutaneous effects under real life conditions for one year.

**Materials and Methods:** Thirty women, 55-65 y, applied either retinol (RO 0.3 %) or retinoic acid (RA 0.025 %) on one forearm dorsal side versus a control product on the other forearm once a day for 1 year. *In vivo* multiphoton imaging was performed every three months and biopsies were taken after 1 year. Epidermal thickness and dermal-epidermal junction undulation were estimated in 3D with multiphoton and in 2D with histology, whereas global melanin density and its z-epidermal distribution were estimated using 3D multiphoton image processing tools.

**Results:** Main results after one year were: i) epidermal thickening with RO (+30 %); ii) slight increase in dermal-epidermal junction undulation with RO; iii) slight decrease in 3D melanin density with RA; iv) limitation of the melanin ascent observed with seasonality within suprabasal layers with both retinoids, using multiphoton 3D-melanin z-epidermal profile.

**Conclusions:** *In vivo* multiphoton imaging and 3D quantification tools allow epidermal effects induced by treatments to being accurately and non-invasively quantified. We also introduce a novel *in vivo* multiphoton 3D descriptor of melanin z-epidermal distribution providing new insights in the knowledge of pigmentation modulation over time with or without treatment.

**Key words:** two-photon excited fluorescence / fluorescence lifetime imaging / human skin / melanin / retinol / retinoic acid / aging / pigmentation

## Introduction

In dermatological clinical research, *in vivo* multiphoton microscopy<sup>1,2</sup> offers the possibility to avoid invasive biopsies and to supply information on the skin state before, during and after a cutaneous treatment. Epidermis and superficial dermis can be characterized with sub-micrometer resolution up to ~160-200  $\mu\text{m}$  depth, by taking advantage of intrinsic multiphoton signals: second harmonic generation (SHG) created by fibrillar collagens and two-photon excited fluorescence (2PEF) emitted by keratin, nicotinamide adenine dinucleotide, flavin adenine dinucleotide, melanin or elastin<sup>3</sup>.

The advent of medically-approved multiphoton microscopes<sup>4</sup> enabled a broad range of clinical applications spanning from the characterization of human skin pigmentation<sup>3,5,6</sup>, age-related or photo-aging changes<sup>7-12</sup>, dermatological disorders and melanoma<sup>13-21</sup> up to the assessment of penetration and effects of pharmaceutical / cosmetic products on human skin<sup>3,12,22-28</sup>.

Multiphoton images of *in vivo* human skin contain a lot of valuable information that can be extracted using appropriate image processing tools. We recently developed the first 3D image processing tools allowing an automatic segmentation of the different skin layers and the extraction of several quantitative parameters<sup>6,10</sup>. For example, epidermal thickness, melanin content or the dermal-epidermal junction (DEJ) shape can be quantified in 3D. Using these tools, we validated several quantitative parameters of interest for studying photo-aging process, constitutive pigmentation or whitening phenomena<sup>3,8,10</sup> and demonstrated that multiphoton-based methods allowed epidermal effects induced by RO and RA to be accurately and non-invasively detected and quantified in the occlusive patch test protocol developed for antiaging products screening<sup>26</sup>. However, this model is not truly representative of the real life situation as occlusion produces effects by itself and of course enhances pharmacological penetration.

The present study aimed at investigating the relevance of *in vivo* multiphoton imaging for the assessment of RO and RA cutaneous effects over one year, under real life conditions, and at calibrating the variation amplitude of multiphoton quantification parameters with these anti-ageing gold standards.

## Materials and Methods

### ***Subjects and treatment***

This study involved 30 healthy European women aged 50-65y with photodamaged skin and a dorsal forearm skin color with ITA value between 10° and 41° (skin color group tanned to intermediate). They applied Retinol 0.3% (RO, Retinol 0.3% cream, L'Oréal group) (n=15) or all-trans-Retinoic acid 0.025% (RA, Retinoic acid 0.025% cream, Galderma) (n=15) on one dorsal forearm versus a control product (white paraffin containing excipient, Bayer) on the other forearm for 1 year, with an every other day application during the first month followed by a daily application for the rest of the study. Additionally, during summer, volunteers were advised to avoid sun exposure, to wear covering sleeves and use a provided sunscreen SPF 50 when exposure to sun was unavoidable. A randomization list, generated by an unblinded data management person, determined the product nature to be applied on the left or right forearm for all volunteers and the study was double blinded, i.e. neither investigator nor volunteers knew the product's nature on each arm.

The study was conducted in Paris, France (February 2011 - April 2012). Evaluations were performed on the same area at months M00 (March), M03 (June), M06

1  
2  
3 (September), M12 (March +1yr) using multiphoton and colorimetric measurements.  
4 Areas of investigation were identified by tracing on transparent plastic sheets  
5 (Monaderm, Monaco) natural anatomic marks (external and internal forearm edges,  
6 ulna head, elbow fold) and also skin folds and neavus on volunteers in a standardized  
7 position. At M12, biopsies were taken for histological analysis. Possible cutaneous  
8 irritation was followed up by a dermatologist at each visit. Recourse to an every other  
9 day application after the first month was allowed if necessary to obtain an acceptable  
10 tolerance.

11  
12 The study was conducted in accordance to the Declaration of Helsinki principles,  
13 approved by Saint-Louis Hospital Ethics Committee and all volunteers gave written,  
14 informed consent (EC reference 2010/58).

### 15 16 **Multiphoton imaging and 3D image processing**

17  
18 Multiphoton imaging was performed with DermalInspect™ (JenLab GmbH, Jena,  
19 Germany), as previously described<sup>25,26</sup>. For each treated area, we acquired two  
20 adjacent 3D-xyz images (z-stacks of 70 *en face* 2PEF/SHG images of 511x511 pixels  
21 (0.25 μm/pixel) acquired with 2.35 μm z-step upon 760 nm excitation). The 2 z-stacks  
22 were acquired within the same central part of the 0.8 cm<sup>2</sup> delimited region over time.

23  
24 The z-stacks were quantified using automatic 3D image processing tools<sup>6,10</sup> to  
25 identify the skin layers, characterize the DEJ 3D-shape and extract quantitative  
26 parameters about the different skin constituents and layers.

27  
28 The first image processing step consists of an epidermis / dermis 3D segmentation,  
29 taking into account the real shapes of skin surface and DEJ, followed by a 3D  
30 epidermis segmentation of SC and LED sub-layers.

31  
32 The second step comprises the extraction of quantitative parameters. We quantified  
33 morphological parameters: SC, LED and total epidermis mean thickness (measured  
34 along the z axis at each (x,y) position and the mean value was computed) and  
35 normalized DEJ area characterizing DEJ undulation in 3D (a generalization in 3D of  
36 the interdigitation index described in 2D in histology<sup>10</sup>). It is expressed as the ratio  
37 between the real DEJ area and the area of its projection on a horizontal plane. Hence,  
38 flat DEJ lead to a ratio of 1 and more undulated DEJ to values above 1.

39  
40 Melanin 3D quantification was performed using Pseudo-FLIM approach combining  
41 multiphoton microscopy and fluorescence lifetime imaging<sup>3,6,12</sup>. Melanin density  
42 corresponds to the ratio between the number of melanin voxels to the number of  
43 epidermal voxels. The epidermal melanin density z-distribution (z-profile of melanin  
44 density in 12 thickness-normalized epidermal layers from 1 - DEJ level to 12 - SC  
45 level) was computed and examples of z-profiles, corresponding raw 2PEF images and  
46 melanin masks are shown in Fig. **Figure 1** **Error! Reference source not found.**

### 47 48 **Colorimetry**

49  
50 Skin color was determined by the ITA values using a microflash spectrophotometer  
51 (Datacolor, Montreuil, France) that was calibrated before each measurement.

### 52 53 **Histology**

54  
55 Two biopsies were collected per volunteer at M12, one on each forearm. Cryostat  
56 sections were processed for light microscopy (NIKON E400) and stained with HES for  
57 overall morphological evaluation, Fontana-Masson, Luna'aldehyde fuchsin and Sirius  
58 red for melanin, elastic fibers and collagen visualization respectively. All  
59 measurements were performed using ImageJ (W. Rasband, NIH, USA).  
60 Measurements of 2D epidermal thickness were taken from the DEJ to the SC surface

1  
2  
3 along the entire section length, and the mean thickness calculated. The 2D  
4 interdigitation index<sup>29</sup>, characterizing DEJ undulation, was calculated as the ratio  
5 between the lengths of basal membrane and *stratum lucidum*, with values closed to 1  
6 revealing a flat DEJ. Quantification of melanin, elastic fibers and collagen 2D densities  
7 correspond to the ratio between the colored surface and the total surface of the  
8 epidermis (melanin) or dermis (elastic fibers and collagen).  
9

### 10 **Statistical methods**

11 For each parameter changes from baseline were analyzed using R. Data distributions  
12 were described for each parameter using boxplots.  
13

14 Results have been interpreted using both effect size (ES) and inferential analysis as  
15 currently recommended<sup>30</sup>. ES represents the difference of means between groups  
16 compared to the variability of the phenomenon and gives an idea of the strength of the  
17 modifications observed between groups. The effect size depends only on the  
18 underlying population parameters, not on the sample size as the *p*-value, and allows  
19 meaningful comparison between different studies outcomes.  
20

21 The criteria for ES interpretation of multiphoton and histology parameters have been  
22 built from contrasts clearly relevant in the study context<sup>26</sup>: very strong:  $ES > 1.3$ ;  
23 strong:  $0.8 < ES < 1.3$ ; moderate:  $0.5 < ES < 0.8$ ; small:  $0.3 < ES < 0.5$ ; very small:  
24  $ES < 0.3$ .  
25

26 For each parameter, the inferential analysis was performed using a linear mixed  
27 model for quantitative longitudinal data with treatment, time, and the interaction  
28 treatment\*time as fixed effects and subject as random. Within and between treatment  
29 groups, comparisons were performed using contrasts. Lsd adjustment for *p*-value tests  
30 was used. All hypothesis tests comparing the treatments were performed using two-  
31 sided tests with  $\alpha = 0.05$  significance level.  
32

33 The results are given in the text as estimated mean  $\pm$  SEM with strength of ES and  
34 *p*-value (N.S. if  $>0.05$ ) in parentheses.  
35

## 36 **Results**

37  
38 In this 1-year kinetic study, control, RO- or RA-treated dorsal forearm skin was  
39 investigated at M00 (March, before treatment), M03 (June), M06 (September) and M12  
40 (March + 1 year) in thirty 55-65 y women using multiphoton imaging and colorimetry,  
41 and at M12 using histology.  
42  
43

### 44 **Quantitation results obtained after 3D image processing of multiphoton images**

#### 45 *Retinoids effects on 3D epidermis thickness*

46 Modifications of living epidermis (LED) and total epidermis (SC+LED) thickness were  
47 similar thus only results on LED are shown (Fig. Figure 2a-b). At baseline, the  
48  $50 \pm 9 \mu\text{m}$  mean LED thickness showed no difference between the two forearms, and  
49 remained unchanged for the study duration on control sites in both groups, except at  
50 M03 for the RO-control group ( $+6,6 \mu\text{m}$ ; *p* N.S., moderate ES).  
51

52 On RO-treated sites, a clear increase in mean LED thickness was shown at M03  
53 ( $+11,1 \mu\text{m}$ ,  $p=0.003$ , strong ES) and M12 ( $+18,5 \mu\text{m}$ ,  $p<0.001$ , very strong ES) as  
54 compared to M00. At M12, LED thickness increase was also significantly higher  
55 compared to control site ( $\Delta 20,5 \mu\text{m}$ ,  $p<0.001$ , very strong ES). The LED thickness  
56 increase is illustrated in Fig. Figure 3 showing multiphoton images and 3D volume  
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3 renderings of the segmented epidermal and dermal compartments at M12 on control  
4 (Fig. Figure 3a) and RO-treated site (Fig. Figure 3b) in a representative subject.

5 On RA-treated sites, slight increase in mean LED thickness was observed at M06  
6 and M12, associated with a moderate ES compared to control but was not statistically  
7 significant ( $p$  N.S.).  
8

#### 9 *Retinoids effects on 3D dermal-epidermal junction undulation*

10 At baseline, the mean DEJ normalized area, characterizing DEJ undulation in 3D, was  
11  $1.7 \pm 0.3$  with no difference between the two forearms and remained unchanged for  
12 the study duration on control sites in both groups (Figure 2e-f). On RO-treated sites,  
13 the mean value increased to  $1.9 \pm 0.3$  at M12 ( $p$  N.S., moderate ES as compared to  
14 M00 and to control). On RA-treated sites, no modification was observed.  
15

16 The dermis surface in Figure 3 allows visualizing the DEJ shape, and hence its  
17 undulation, within the  $\sim 130 \times 130 \mu\text{m}^2$  field of view.  
18

#### 19 *Modulation of global epidermal 3D melanin density with seasonality and retinoids*

20 At baseline (Figure 4a), the mean 3D epidermal melanin density was  $14 \pm 0.1 \%$ , with  
21 no difference between the two forearms in both groups. At M06, after summer, a clear  
22 increase of 9 to 15% is observed, on control and retinoid-treated sites, in both groups  
23 as compared to M00 ( $p < 0.005$  with strong to very strong ES for all conditions), with no  
24 difference between the control and retinoid-treated sites. At M12, melanin density in  
25 the control of RO-group decreased back to M00 level, whereas in the control of RA-  
26 group, a persistent increase was observed compared to M00 (+7%  $p = 0.019$ , strong  
27 ES). In the treated conditions at M12, melanin density decreased back to M00 level for  
28 both treated sites with no difference compared to control in RO-treated sites, but with  
29 a slight difference in RA-treated sites compared to control (-6%,  $p$  N.S., moderate ES).  
30  
31

#### 32 *Modulation of 3D melanin z-epidermal distribution with seasonality and retinoids*

33 When dividing the epidermis into several thickness-normalized sub-layers, one can  
34 measure the 3D melanin z-epidermal distribution profile and get an insight into melanin  
35 content changes appearing within these layers: synthesis within the basal layer or  
36 transfer within the upper layers. Depending on the modulation level of this profile, the  
37 changes can impact or not the global epidermal 3D melanin density. For example, a  
38 decrease in one layer could be counteracted by an increase in another layer and hence  
39 no change will be evidenced in the global epidermal 3D melanin density. Also, slight  
40 modulations not detectable at the global level can be evidenced.  
41

42 In the control condition (Figure 4c left), analysis of melanin z-epidermal distribution  
43 revealed that the gradual increase of global 3D epidermal melanin density is due to an  
44 increase in the basal layers, but also up to the middle epidermis at M03, and throughout  
45 the epidermis at M06 (after summer). This can also be visualized in Figure 4b, on the  
46 M00 and M06 3D melanin masks of a representative control subject. At M12, a  
47 persistent accumulation of melanin in the basal and supra-basal layers, as well as at  
48 the SC level was observed when compared to M00.  
49

50 In both retinoid-treated groups (Figure 4c right), the strongest modulation of melanin  
51 distribution induced by summer (M06) wasn't different compared to control group, but  
52 the ascent of melanin in the supra-basal layers appearing in control groups wasn't  
53 observed at M03 nor at M12. The z-profile of melanin density at these times overlaps  
54 M00 curve for RO-condition and even slightly decreased in the first third of epidermis  
55 for RA-condition at M12 consistent with the slight difference in global epidermal  
56 melanin density observed at this time.  
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3 Examples of multiphoton 2PEF images, melanin masks and 3D melanin z-epidermal  
4 distributions at M12 on control and RA-treated site in a representative subject are given  
5 in Fig. **Figure 1** **Error! Reference source not found.**  
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### 8 **Results from histology**

9 At M12, consistent with multiphoton results, quantitative measurements of the HES-  
10 stained biopsy skin sections showed following treatment with RO and RA a mean  
11 increase in epidermal thickness by 19.6  $\mu\text{m}$  ( $p=0.002$ , very strong ES) and 10.4  $\mu\text{m}$   
12 ( $p$  N.S., moderate ES) respectively compared to control (Figure 2c-d). Increase in DEJ  
13 undulation was not statistically significant, but showed a moderate ES on RO-treated  
14 site compared to control (Figure 2g-h). Figure 5 **Error! Reference source not found.**  
15 shows representative histologic photomicrographs of control and retinoid-treated sides  
16 at M12. Histological quantification of melanin, elastic fibers and collagen showed no  
17 statistically significant difference between retinoid-treated and control sites at M12  
18 (data not shown).  
19  
20

### 21 **Results from colorimetric measurements**

22 At baseline, the mean ITA value was  $38 \pm 7$  for all conditions, and decreased of about  
23  $5^\circ$  at M03 ( $p<0.015$ , moderate ES for all conditions, except RA-treated site  $p$  N.S.,  
24 weak ES) and  $10^\circ$  at M06 ( $p<0.001$ , strong to very strong ES), indicating moderate  
25 tanning, before increasing to the baseline value again at M12 in all conditions with no  
26 differences between them (data not shown).  
27  
28

### 29 **Tolerance**

30 Despite an every other day application during the 1<sup>st</sup> month, erythema or mild burning  
31 or stinging were noted in 14 RO- and in 6 RA-treated volunteers, mostly transient and  
32 rated as mild. For 5 subjects, the number of planned applications was reduced and  
33 additional every other day application periods prescribed: for 1 month (1RA, 1RO), 4  
34 months (1RO) and for all the study duration (2RO). One subject was released from the  
35 study at M06 (RO).  
36  
37  
38

## 39 **Discussion**

40 Despite its small field of view, long image acquisition time, cost and the expertise  
41 required to master the technology, *in vivo* multiphoton imaging affords quantitative  
42 information on human skin constituents with unprecedented specificity. This study  
43 confirms the capabilities of multiphoton imaging combined with specific 3D image  
44 processing tools for time-course assessment of dermatological/cosmetic treatments.  
45 Indeed, in agreement with literature<sup>31-37</sup>, RO and RA are shown to increase epidermal  
46 thickness after one year with both 3D-multiphoton and 2D-histological quantifications.  
47 Regarding DEJ undulation, we previously reported with multiphoton imaging an  
48 increase of this parameter with 0.3% RO in the occlusive patch test<sup>26</sup>. In this study,  
49 although non statistically significant, a slight increase is detected with RO, associated  
50 with moderate ES with both multiphoton (versus M00 and versus control) and histology  
51 (versus control) after 1 year. The beneficial effects on epidermis are here more  
52 pronounced with RO than with RA and this is likely due to the high concentration of RO  
53 used (0.3%), 12 times higher compared to RA concentration (0.025%), as in our  
54 previous short term study<sup>26</sup>.  
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59 Beside epidermal morphological changes, multiphoton fluorescence lifetime  
60 imaging has the unique capacity to specifically detect, quantify and describe the

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3 precise 3D-distribution of melanin in the epidermis<sup>3</sup>. The study shows *in vivo* the  
4 progressive increase of global 3D-melanin density with summer. It's noteworthy that  
5 the summery modulations of melanin density are quite weak in this study, due to the  
6 sun-protection regime prescribed to the volunteers. The instructions were overall  
7 respected, as evidenced by a rather low increase of pigmentation in summer as  
8 measured by a mean decrease of ITA of 10° which corresponds to the usual difference  
9 of colour observed between the dorsal and the ventral side of the forearm in winter in  
10 healthy subjects of the same age and phototype (personal data).  
11

12 This study also reveals for the first time, the gradual ascent of melanin from the  
13 basal layers into the upper epidermal layers. This melanin z-epidermal distribution  
14 allows identifying small changes that are not necessarily detectable or associated to a  
15 change at the global level. Indeed, on control sites after one year, this melanin z-profile  
16 reveals a persistent melanin in the supra-basal layers, in the first third of epidermis.  
17 The same pattern of melanin distribution has also been observed in other studies that  
18 compared either, chronically exposed and non-exposed skin in the same subject, or  
19 old vs younger subjects on the same chronically photo-exposed area at distance of  
20 seasonality effect (personal data). Thus persistent melanin into the supra-basal layers  
21 of epidermis could be a new pertinent descriptor of photoageing in Europeans. Its  
22 advantage over other photoageing markers is that its modulation is evident over one  
23 year, whereas flattening of DEJ or epidermal thinning are not detectable in such a short  
24 time frame. Moreover, the improvement seen with gold standard antiageing treatments  
25 validates this parameter as a good candidate for further anti-photoageing products  
26 evaluation. Indeed, in both retinoid-treated conditions, the progressive ascent at M03  
27 and persistent ascent at M12 in pigmentation, appearing within supra-basal epidermal  
28 layers of control groups, is not observed.  
29

30 This visualization of the modulation of global melanin content and its epidermal  
31 distribution under treatment also strongly supports that *in vivo*, the predominant effect  
32 of retinoids on pigmentation is more likely due to skin renewal than to a direct effect on  
33 melanogenesis as discussed in literature<sup>38-40</sup>.  
34

35 As already shown<sup>8,10</sup>, multiphoton imaging also allows investigating human skin up  
36 to ~150 µm depth corresponding to approximately 80 µm of superficial dermis in a  
37 normal untreated skin. In this study, the biggest proportion of dermis between treated  
38 and control skin at every time point was restricted to a 30 µm thick dermal layer below  
39 the DEJ. This relates to the mean epidermal thickening of 30% in RO-treated areas,  
40 even higher than 50% in a third of volunteers of this group. In these conditions, the  
41 superficial dermis was not assessed by multiphoton. After one year, no obvious  
42 quantitative change in the dermal fibrillary networks was shown by standard  
43 histological assessment in our study. Although retinoids are known to increase  
44 expression of type I procollagen<sup>36,41-43</sup> a direct increase of collagen by retinoids has  
45 rarely been demonstrated and a decrease of dermal elastosis has only been observed  
46 after up to 4 years therapy<sup>33</sup>. Hence, we believe that changes in the dermis that could  
47 be addressed with this technique are not likely to be observed in such study duration.  
48  
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51

## 52 Conclusion

53 This study shows for the first time that, under real life conditions, *in vivo* multiphoton  
54 microscopy associated with specific 3D quantification tools allows epidermal effects  
55 induced by treatments, including melanin content, to being accurately and non-  
56 invasively quantified. *In vivo* melanin z-epidermal distribution is assessed here for the  
57 first time and provides new insights in the knowledge of pigmentation modulation with  
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3 seasonality and retinoids treatment. Knowing the importance of epidermal melanin  
4 distribution for its DNA protection factor<sup>44-46</sup>, our new method could be applied to  
5 improve the knowledge of some underlying biological mechanisms of pigmentation  
6 modulations appearing through either redistribution of existing melanin and/or *de novo*  
7 melanin synthesis. The applications of these multiphoton-based melanin quantification  
8 parameters can span from physiological, pathological or environmental factors-  
9 induced pigmentation modulations up to whitening, anti-photoageing or  
10 photoprotection products evaluation.  
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## Figures

**Figure 1** Pseudo-FLIM melanin quantification and melanin density z-profile. (top) Examples of *in vivo* 2PEF raw multiphoton images (cyan hot colour) with corresponding melanin masks, obtained after image processing using Pseudo-FLIM approach, at different depths within the epidermis of a) control and b) retinoic acid-treated skin at M12. (bottom) Corresponding melanin z-epidermal distribution profiles (melanin density in 12 thickness-normalized epidermal layers from 1 - DEJ level to 12 - SC level). SC – *stratum corneum*, SG – *stratum granulosum*, SS – *stratum spinosum*, DEJ – dermal-epidermal junction.

**Figure 2** Multiphoton and histology quantification results of retinol 0.3% and retinoic acid 0.025% effects on epidermis thickness and dermal-epidermal junction undulation over one year. (a, b) Living epidermis thickness measured in 3D by multiphoton microscopy; (c, d) epidermis thickness measured in 2D by histology; (e, f) DEJ undulation measured in 3D by multiphoton microscopy; (g, h) DEJ undulation measured in 2D by histology. No unit (ratios), both parameters equal to 1 for a totally flat DEJ and >1 for a more undulated junction (see Materials and methods section). The data are expressed as boxplots with fences.

**Figure 3** *In vivo* multiphoton images and 3D volume renderings of retinol 0.3% effects at M12. (a) control and (b) RO-treated side in a representative subject. The mosaic images show all the images within a z-stack of combined 2PEF (cyan hot colour) / SHG (red colour) images. The images in the middle correspond to their respective 3D volume reconstruction of the segmented epidermal and dermal compartments, obtained using the 3D automatic segmentation method and allowing to visualize epidermis thickness and DEJ shape. The epidermis is depicted as a cyan bracket and the dermis as a red bracket. The 3D volume reconstructions were created using ImageJ software.

**Figure 4** Modulation of melanin global density and z-epidermal distribution with seasonality and retinoids. a) change with time in the global 3D epidermal melanin density. The data are expressed as boxplots with fences. b) 3D melanin masks of a representative control subject at M00 and M06. These 3D reconstructions were created with Imaris (Bitplane AG, Zürich, Switzerland) software; c) Melanin z-epidermal distribution (mean 3D melanin density estimated in 12 thickness-normalized epidermal layers from 1 - DEJ level to 12 - SC level). The z-profiles data are expressed as mean  $\pm$  SEM.

**Figure 5** Histologic photomicrographs of control and retinoid-treated skin in representative subjects at M12. HES (Hematoxylin-eosin stain, x20) images of a) control of RO-group, b) RO-treated skin, c) control of RA-group and d) RA-treated skin in representative subjects at M12.

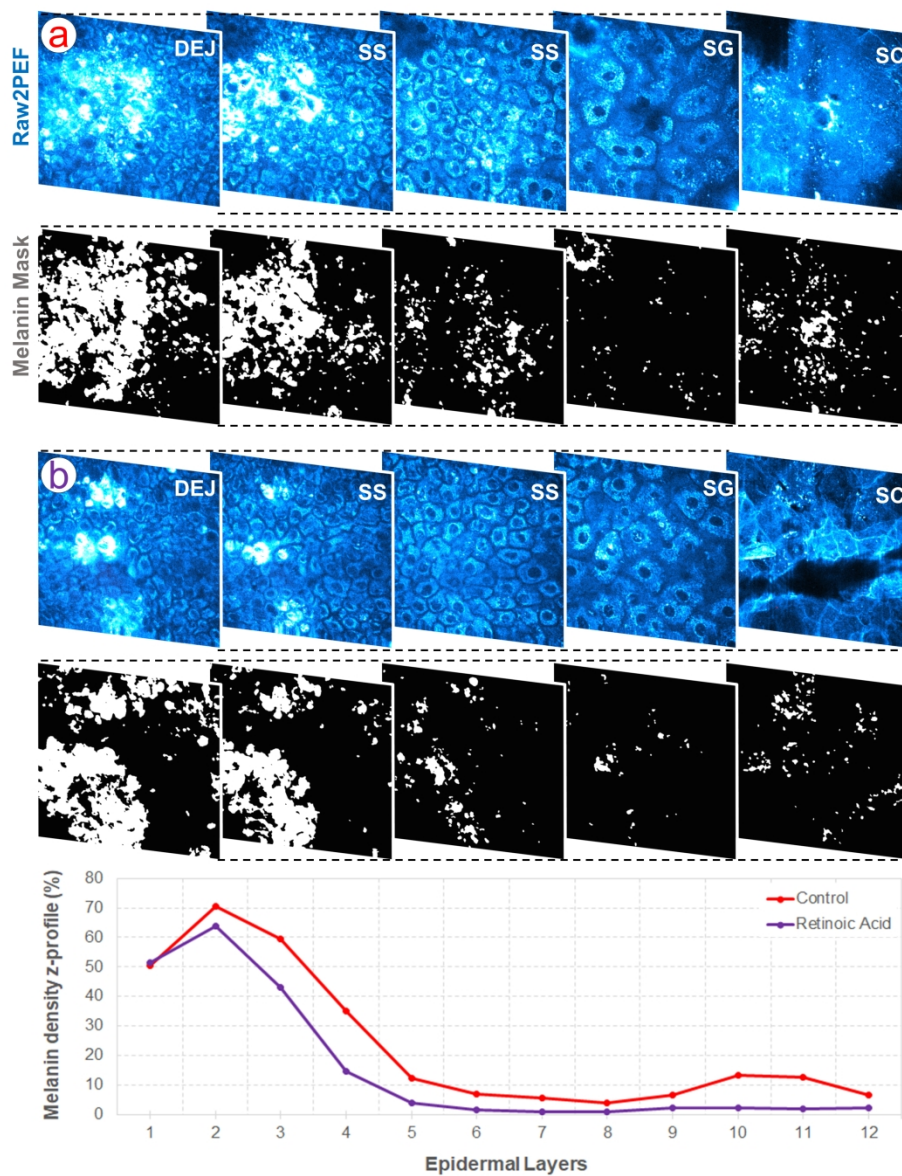


Figure 1. Pseudo-FLIM melanin quantification and melanin density z-profile. (top) Examples of in vivo 2PEF raw multiphoton images (cyan hot colour) with corresponding melanin masks, obtained after image processing using Pseudo-FLIM approach, at different depths within the epidermis of a) control and b) retinoic acid-treated skin at M12. (bottom) Corresponding melanin z-epidermal distribution profiles (melanin density in 12 thickness-normalized epidermal layers from 1 - DEJ level to 12 - SC level). SC - stratum corneum, SG - stratum granulosum, SS - stratum spinosum, DEJ - dermal-epidermal junction.

800x1050mm (96 x 96 DPI)

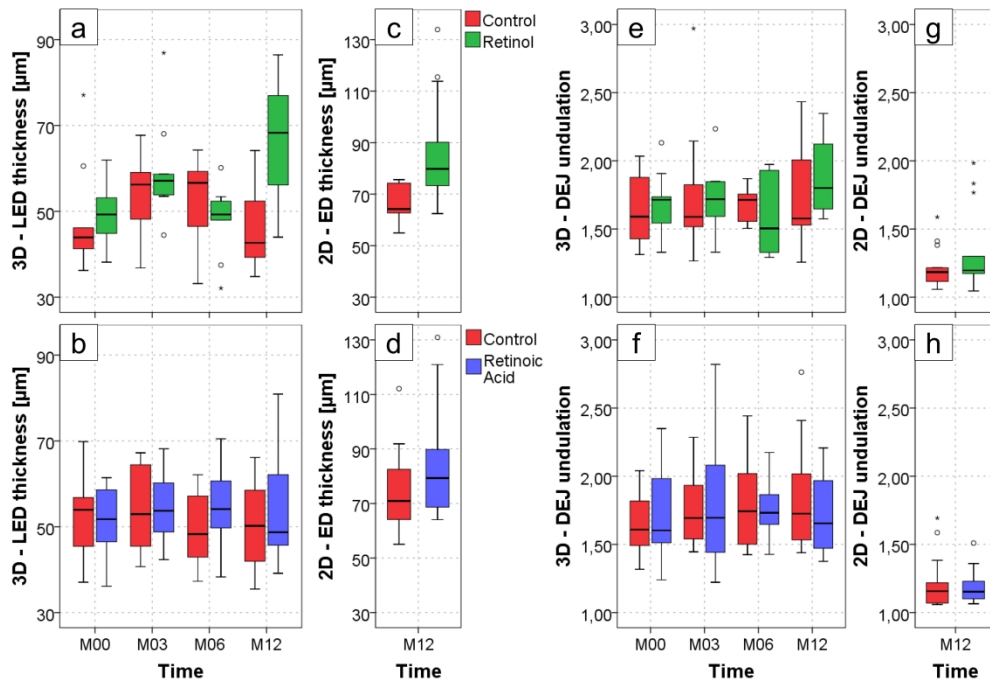


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1219x850mm (96 x 96 DPI)

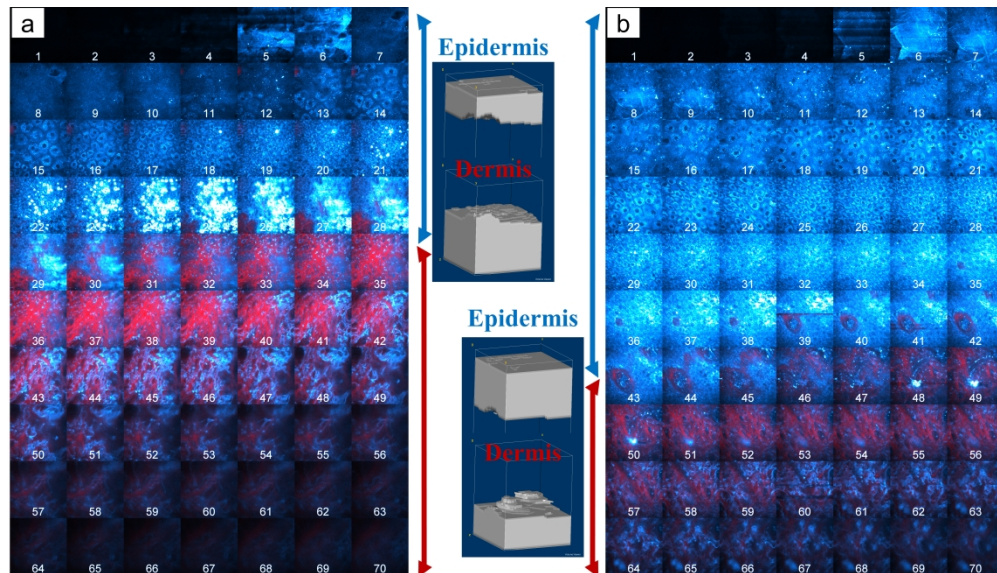


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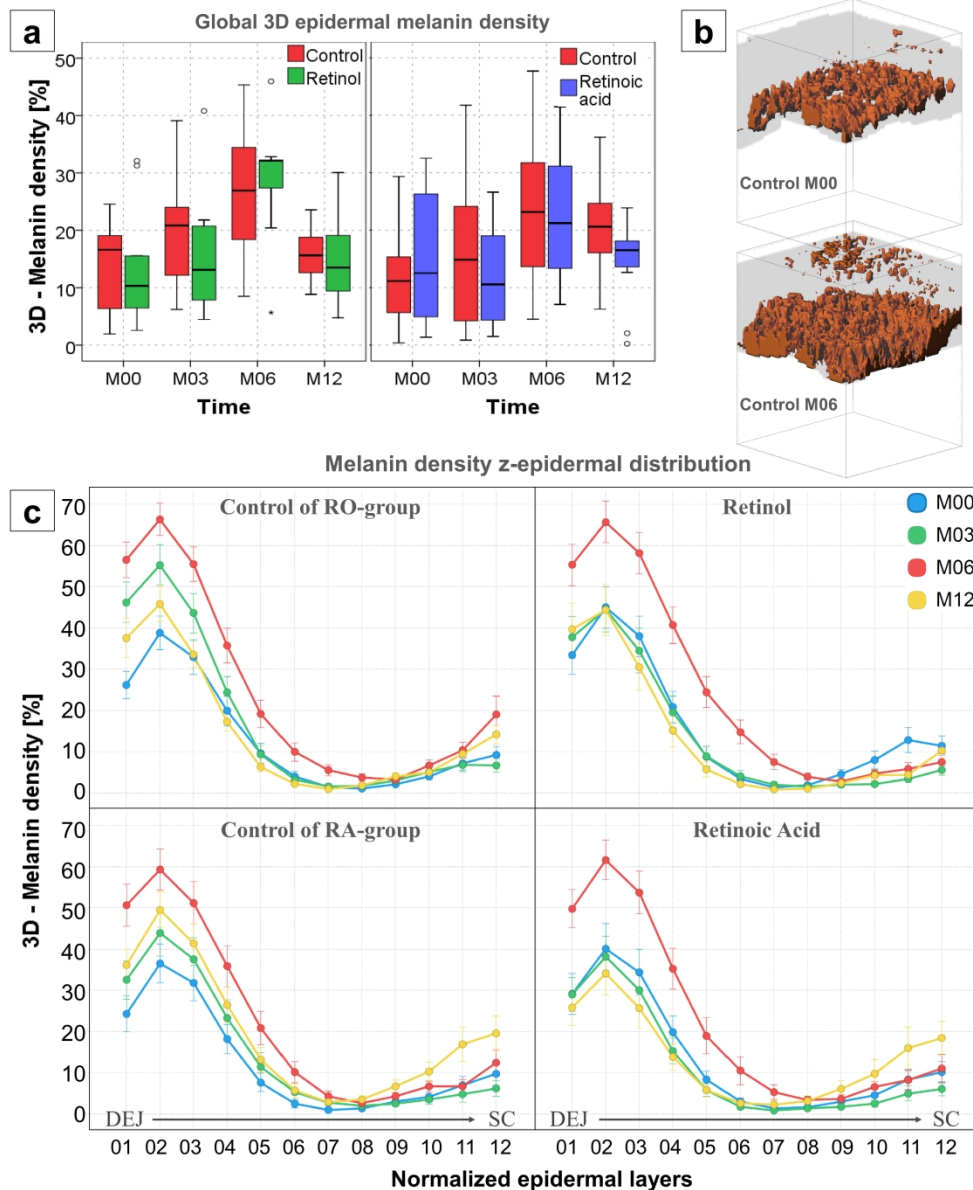


Figure 4. Modulation of melanin global density and z-epidermal distribution with seasonality and retinoids. a) change with time in the global 3D epidermal melanin density. The data are expressed as boxplots with fences. b) 3D melanin masks of a representative control subject at M00 and M06. These 3D reconstructions were created with Imaris (Bitplane AG, Zürich, Switzerland) software; c) Melanin z-epidermal distribution (mean 3D melanin density estimated in 12 thickness-normalized epidermal layers from 1 - DEJ level to 12 - SC level). The z-profiles data are expressed as mean  $\pm$  SEM.

1030x1249mm (96 x 96 DPI)

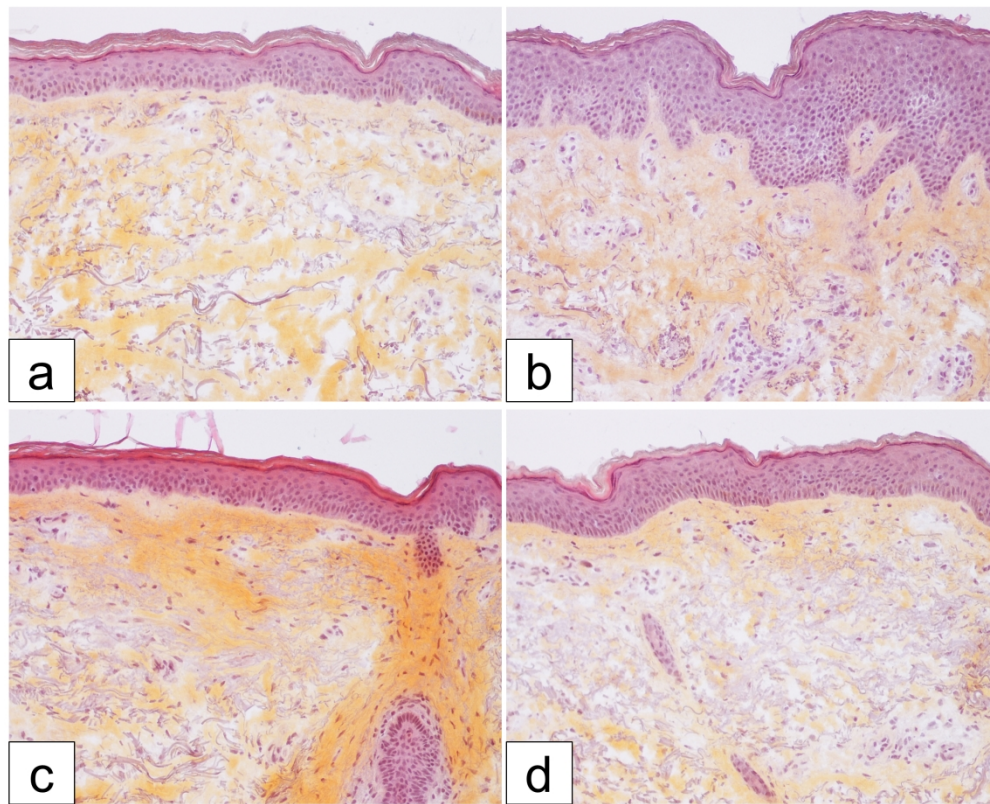


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700x560mm (96 x 96 DPI)