Investigating melanin and its distribution in reconstructed pigmented epidermis model by fast 2D XZ multiphoton imaging

LIU Juan[1], CHEN Juanjuan[1], CASTIEL Isabelle[3], PENA Ana-Maria[2], FU Yinfeng[1], MALHERBE Emmanuel[1], Ding Chunmei[1], ZANG Shanshan[1*]

[1] L'Oréal Research and Innovation, NO.550 Jinyu Road, Pudong District, 201206, Shanghai, China
[2] L'Oréal Research and Innovation, 1 avenue Eugène Schueller, BP22, 93601, Aulnay-sous-bois, France
[3] L'Oréal Research and Innovation, CCZ-Rio-9 Rue Dreyfus, 92110 Clichy, France

* ZANG Shanshan, NO.550 Jinyu Road, Pudong District, 201206, Shanghai, China, +86-18512124102，sszang@rd.loreal.com

Abstract (Maximum of 250 words)

Background: Investigating melanin's density and z-epidermal distribution in skin is essential for medical and cosmetic applications. The *in situ* characterization of this skin pigment requires the use of label free non-invasive imaging methods such as multiphoton microscopy.

Methods: We applied melanin fast 2D XZ multiphoton imaging method and image processing tool to the study of melanin distribution in reconstructed pigmented epidermis (RPE) model upon application of different associations of Ferulic Acid, Niacinamide and Symwhite (Phenylethyl Resorcinol), and compared the results to control samples.

Results: The results show a stronger decrease in melanin density for the association of Ferulic Acid, Niacinamide and Symwhite (Phenylethyl Resorcinol) compared to placebo and to Symwhite (Phenylethyl Resorcinol) and Niacinamide association.

Conclusion: The association of fast 2D XZ multiphoton imaging with specific image processing tool allows the distribution and quantity of melanin to be visualized and quantified in RPE epidermis model with an operational time 5 times faster compared to 3D imaging, thus providing a powerful method for routine efficacy evaluation of melanin modulators in cosmetic and dermatological investigations.

Keywords: multiphoton, melanin, quantification, reconstructed pigmented epidermis, two-photon excited autofluorescence.

Introduction.

Investigating melanin's density and z-epidermal distribution in skin is essential for medical and cosmetic applications. The *in situ* characterization of this skin pigment requires the use of label free non-invasive imaging methods such as multiphoton microscopy. Using endogenous autofluorescence signals from melanin, keratin, NAD(P)H and FAD metabolic coenzymes, multiphoton microscopy provides non-invasive 3D visualization of epidermal constituents and allows assessing melanin's distribution *in vivo* in human skin[1-7] and *in vitro* in reconstructed pigmented epidermis (RPE) model[8]. We previously reported on a multiphoton-based method for 3D quantitative assessment of melanin content in RPE model[8], however 3D imaging is very time consuming and greatly limits its routine use for efficacy evaluation of melanin modulators.

In this study, we report on a new faster method for melanin assessment in RPE model, based on the acquisition of several transversal 2D XZ multiphoton images, equivalent to the ones provided by histology Fontana-Masson (FM) staining. We applied melanin fast 2D XZ multiphoton imaging method to the study of melanin modulations in RPE model upon application of different associations of Ferulic Acid, Niacinamide and Phenylethyl Resorcinol, and compared the results to control samples.

Materials and Methods.

1. Reconstructed pigmented epidermis (RPE) model

Pigmented epidermis model was reconstructed according to the technique described previously[8]. Briefly normal human keratinocytes (NHK) and melanocytes (NHM) were seeded onto an insert with a bovine collagen matrix as dermal substitute. After an immersion culture for keratinocyte proliferation, cells were exposed to an air–liquid interface to sustain keratinocyte differentiation. Then, all the tissue samples were incubated at 37°C with 5% CO_2 and saturated humidity.

2. Raw material preparation

For all the raw materials (RM) to be evaluated, we first prepared the stock solutions in their appropriate solvents (DMSO, water). The working solution is made by diluting the stock solution into the culture medium. Normally, the dilution is calculated in order that the solvent concentration in the medium is always equal to 0.1% (DMSO as solvent) and 1% (water as solvent).

3. Systemic application of ingredients on RPE models

The RPE models are treated by adding tested RM or solvent control to the culture medium starting from day 9 and changed with freshly prepared culture medium each day until day 17 (D9~D17, 6 applications). For each experimental condition, 6 RPE samples were investigated and a portion of each sample was used for histology and imaging analyses (1/4 of epidermis for DOPA; 1/2 of epidermis for hematoxylin eosin (HE) and Fontana-Masson (FM) staining) and 1/4 of epidermis for multiphoton imaging).

4. Histology analysis of melanocytes morphology in RPE model

The RPE samples were cut into 2 halves: one was fixed in 10% formaldehyde and processed for embedding in paraffin for histological sections. Two 5 µm-thick transverse (vertical) sections were prepared for HE and respectively FM staining and alanysed using transmitted light microscopy. The other half was used for DOPA staining. The reconstructed epidermis was separated from the dermal support and put in 1% L-DOPA solution after formalin fixation. By the action with tyrosinase in the melanosomes, the L-DOPA is oxidized to DOPA-quinone and presents black intra-melanosomal coloration which allows for the visualization of melanocytes morphology.

5. Melanin quantification by Fontana-Masson histology analysis

The melanin present in RPE is stained with Fontana-Masson staining on 5µm-thick transverse sections, then quantified by image analysis. Every epidermis slide is scanned using Nanozoomer® system (HAMAMATSU, Japan). For each epidermis, 10 to 15 images (1920*1200 pixels (0.45 µm/pixel)) were acquired within different regions of the section (transmitted light microscopy, magnification x 20). The area occupied by melanin (pixels with black color) was quantified using Histolab® software (Microvision, Evry, France).

Descriptive statistics and statistical analysis (non-parametric Mann-Whitney U test, n=6) were performed using SPSS® software (IBM, New York, United States). The data were expressed as box plots with fences and median. For each sample, the data from the 10 to 15 images were averaged and for each experimental condition, the 6 values were considered for analysis. The significance threshold for comparisons was set at 5% (*p*-values:*$\leqslant$0.05).

6. Multiphoton microscopy analysis

Multiphoton imaging was performed with an up-right laser scanning microscope (Nikon A1RMP/FN1, Tokyo, Japan). Endogenous fluorescence 2PEF signals were excited by a fs pulsed laser (MaiTai Deepsee, Spectra-Physics Santa Clara, California) adjusted to 760 nm, with typical 8 mW power at the sample. For high-resolution imaging, a high numerical-aperture, water immersion objective (25x, 1.1 NA, Nikon) was employed in all experiments. For each sample, 50 XZ images (1024x1024 pixels; dx=0.497 µm; dz=0.410 µm/pixel) were acquired every dy=100 µm. Melanin quantification was performed using ImageJ software (W. Rasband, NIH, USA). We also recorded one 509x509x100 μm^3 z-stack (101 images of 1024x1024 pixels; 0.497µm/pixel; dz=1 µm) for each sample for 3D visualization purposes (Fig1a). All 2PEF images were acquired from *stratum basale* to *stratum corneum.* Melanin is identified in the living epidermis by its high autofluorescence intensity compared to the other endogenous constituents (Fig1b). The 3D image reconstructions were done using NIS Element software (Nikon, Tokyo, Japan).

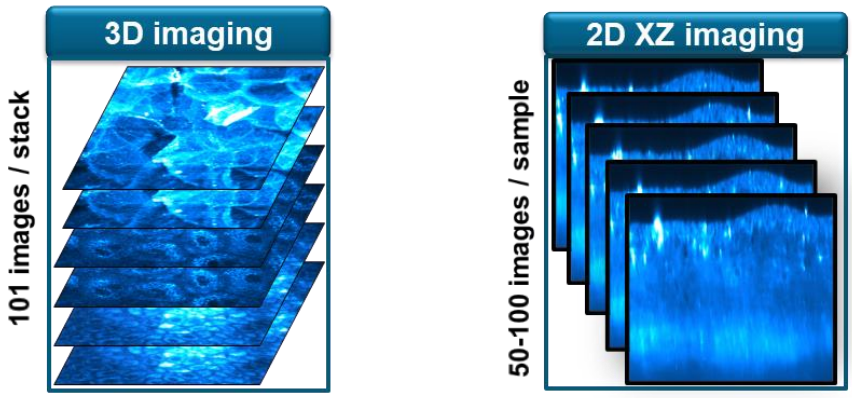

Fig1. Multiphoton microscopy working flow

7. Multiphoton melanin distribution analysis

All 50 multiphoton transverse XZ images are saved as a stack in 'tiff' format. After that we need to use the cross section, which is alongside the Y axis, as the calculation layer to sum up the melanin pixels and obtain the distribution from the basal skin to the deeper. In this case, all work was implemented in Python using Numpy and OpenCV packages.

First, we do coding to read and traverse all the tiff images and split into single layers for next processing step. Second, because this is a real-world problem, there are several factors to be considered: the skin cannot be flat, and the variation could differ in each image during when it was taken, it is difficult to find the exactly original boundary which means the surface of the skin. Therefore, we had to implement boundary detection to find the border between the air and the skin for each single layer we obtained above. In order to reduce noise on the image, since the intensity of the skin pixels will be much higher than those of the air, we binarize the picture by applying threshold in pixels intensity. Then for each sliding interval in the x axis, we regroup the y-values of each first positive pixels in vertical lines. We consider the skin point of this x-value as a specific percentile of those y-values. In other terms, we define this signal point as the start of the skin, which means there is a mutation from the air to the skin. After concatenating all these values into a smooth curve, we get the Boundary line. In the third step, we detect the melanin. To do so, we use a Gaussian blur to further eliminate noise and apply another threshold for binarization in order to identify the melanin masses. This is equivalent to the signal pixels is the initial part, on account of the melanin pixels have a higher intensity than others. After that, we can use the previously detected boundary as the starting point in y-axis, so that melanin pixels can be then counted with respect to their distance from the skin boundary. We implement this action for 256 pixels following the start point and aggregate all the values layer by layer. By averaging this quantity for every layers, we get the melanin distribution from basal to the deeper for one tiff image.

Finally, we sum up the counting results of all acquisitions of one group and take the average curve. By doing so we obtain 3 melanin distribution curves for 3 different groups, which can be plotted and reflect the trend how melanin changed with skin depth. By computing and comparing among those groups, including two experimental groups using the products and a

control group, we acquire a result which shows a decreasing trend in percentage to prove the effectiveness of our products in reducing melanin.

Results.

Histology DOPA analysis of melanocytes morphology in pigmented epidermis model

After treatment with cosmetic ingredients (actives), the cytotoxicity of the treatment was estimated by analyzing the tissular structure on HE images (HE notation) and the morphology of melanocytes using DOPA notation. We observed no significant cytotoxicity after the application, consistent with the solvent control.

The cytotoxicity results are shown in Fig. 2 and 3.

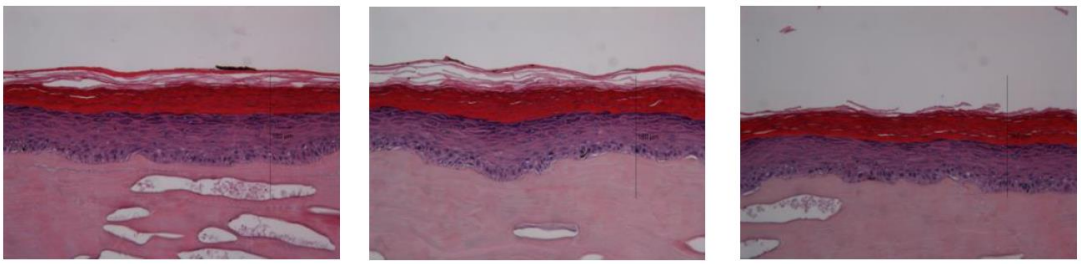

Fig2. HE staining images

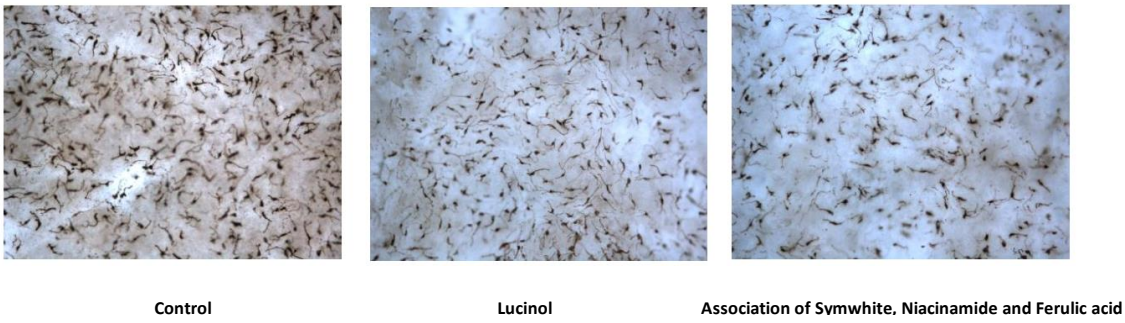

Fig3. DOPA staining images

Melanin quantification by Fontana-Masson image analysis

From the Fontana-Masson image analysis results below, we can find that Rucinol presented significant depigmentation activity at 0.0005% compared to DMSO. Both associations of active ingredients showed significant depigmentation effect compared to DMSO. The depigmentation effect of the association of Symwhite (Phenylethyl Resorcinol), Niacinamide

and Ferulic acid is significantly stronger than the association of Symwhite (Phenylethyl Resorcinol) and Niacinamide. (Fig 4 & Table 1)

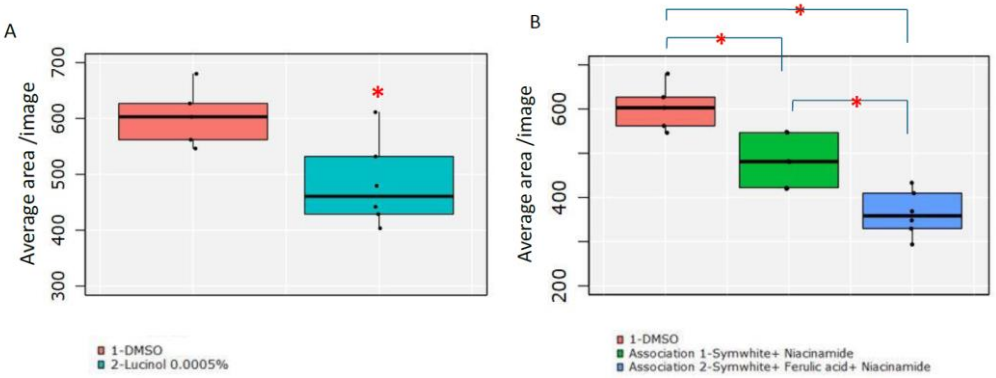

Fig 4. Melanin quantification results by Fontana-Masson image analysis

A. The depigment effect of Lucinol compared to solvent control

B. The depigment effect of 2 associations compared to solvent control and the comparison between 2 associations.

TOPICAL	Placebo	Lucinol 0.0005%	Association 1 （symwhite 0.0006%+Niacinamide 0.0019%）	Association 2 （symwhite 0.0006%+Niacinamide 0.0019%+Ferulic acid 0.001%）
Average area/image	604.12	480.9	483.12	363.23
P-value (Mann-Whitney)		0.028	0.018	0.006

Table 1. Statistical analysis results of melanin quantification by Fontana-Masson image analysis.

Melanin imaging and quantification by multiphoton microscopy

Fig 5 shows representative images of the melanocytes and melanin (green) present within the reconstructed skin as visualized by 3D multiphoton imaging, with the depigmenting effect of the associations 1 and clearly visible. This effect was also quantified based on melanin fluorescence with fast 2D XZ multiphoton imaging, similarly demonstrating a statistically-significant depigmentation in association-treated samples (Fig 6 &Table 2).

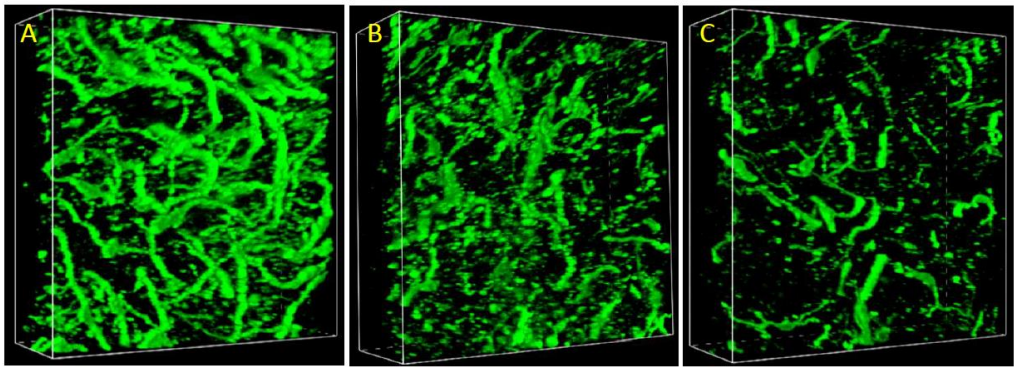

Fig 5. Representatives multiphoton 3D images of RPE model treated with A: Solvent control (DMSO), B : Association 1, C: Association 2

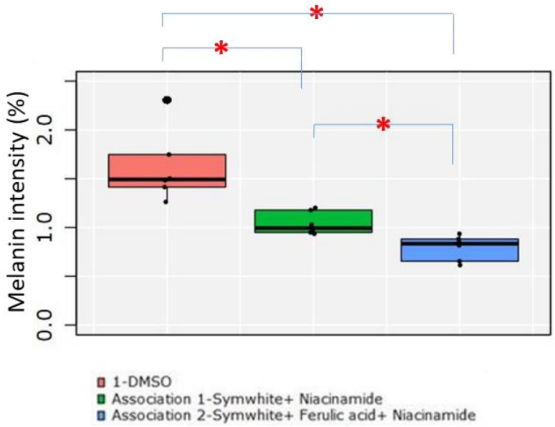

Fig 6. Epidermal melanin density (%) results by fast 2D XZ multiphoton microscopy

TOPICAL	Placebo	Association 1 (symwhite 0.0006%+Niacinamide 0.0019%)	Association 2 (symwhite 0.0006%+Niacinamide 0.0019%+Ferulic acid 0.001%)
Melanin intensity (%)	1.619	1.042	0.792
P-value (Mann-Whitney)		0.004	0.004

Table 2. Statistical analysis results of melanin quantification by fast XZ multiphoton microscopy.

Melanin z-epidermal distribution results using the new image analysis algorithm

Since we got the melanin distribution curve, we can obtain the amount of melanin contained and analyze its changes by integrating it. After integrating, we can see that (Fig 7):

- In the shallow layer (pixel 0 to 40), the melanin content of Association 1 is 13.28% lower than that of Placebo, and Association 2 is more significantly lower compared to

Placebo, of which the improvement reached 40.55%. While Association 2 is 31.44% lower compared to Association 1.

- In the deeper layer (pixel 40 – 256), as the same way, Association 1 is 2.33% lower than Placebo, while Association 2 is 26.49% lower. Meanwhile, in this skin layer, Association 2 decreased by 24.74% compared to Association 1.
- The aforementioned results demonstrated the effectiveness of our products in reducing melanin content in RPE model.

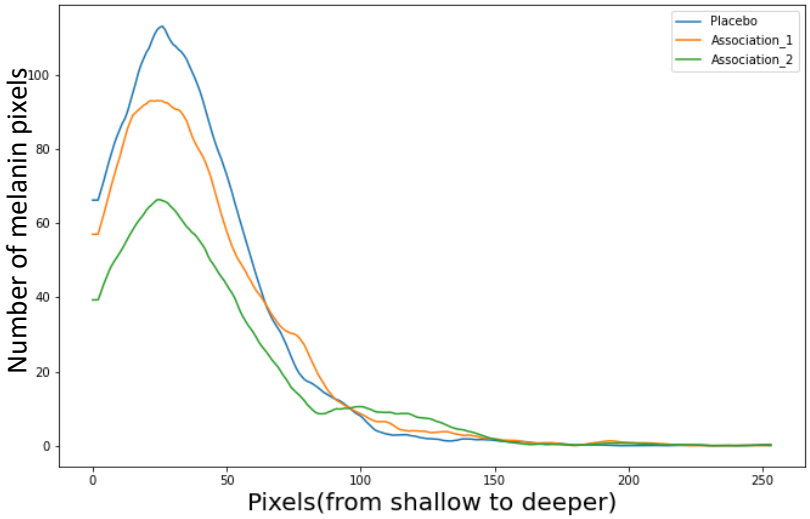

Fig 7. Melanin z-epidermal distribution results obtained by fast XZ multiphoton imaging

Discussion.

This study demonstrated that association 1 (Symwhite (Phenylethyl Resorcinol)and Niacinamide) and association 2 (Phenylethyl Resorcinol, Niacinamide and Ferulic acid) led to a decrease in melanin content in the reconstructed pigmented epidermis by fast 2D XZ multiphoton microscopy analysis, consistent with the results from Fontana-Masson image analysis. Using fast 2D XZ multiphoton imaging method, one can acquire representative images of melanin density within a larger skin area, and at an operational time 5 times faster compared to 3D imaging. Multiphoton XZ imaging combined with the newly developed algorithm for melanin distribution analysis allow for an optimized *in vitro* multiphoton melanin assessment workflow and it brings a great improvement not only for enhancing the research accuracy during the experiments, but also for significantly improved processing

speed in large-scale image processing, strongly assisting experimental progress. Experiments that used to take three days to complete now only take 5 minutes to produce results, which is about 860 times more efficient.

Conclusion.

The association of fast 2D XZ multiphoton imaging with specific image processing tool allows melanin density and z-epidermal distribution to be visualized and quantified in pigmented epidermis model with an operational time 5 times faster compared to 3D imaging, thus providing a powerful method for routine *in vitro* efficacy evaluation of melanin modulators in cosmetic and dermatological investigations.

Conflict of Interest Statement.

NONE.

References.

1 Pena, A.-M., Baldeweck, T., Tancrede, E., Decencière, E. & Koudoro, S. Non-invasive method for specific 3D detection, visualization and/or quantification of an endogeneous fluorophore such as melanin in a biological tissue. France patent French patent FR2982369, International publication number WO2013068943 (2011).

2 Ait El Madani, H. *et al. In vivo* multiphoton imaging of human skin: assessment of topical corticosteroid-induced epidermis atrophy and depigmentation. *J Biomed Opt* **17**, 026009, doi:https://doi.org/10.1117/1.JBO.17.2.026009 (2012).

3 Decenciere, E. *et al.* Automatic 3D segmentation of multiphoton images: a key step for the quantification of human skin. *Skin Res Technol* **19**, 115-124, doi:https://doi.org/10.1111/srt.12019 (2013).

4 Tancrede-Bohin, E. *et al.* Non-invasive short-term assessment of retinoids effects on human skin in vivo using multiphoton microscopy. *J Eur Acad Dermatol Venereol* **29**, 673-681, doi:https://doi.org/10.1111/jdv.12650 (2015).

5 Pena, A.-M. *et al. Multiphoton FLIM in cosmetic clinical research* in *Multiphoton Microscopy and Fluorescence Lifetime Imaging. Applications in Biology and Medecine* (ed Karsten König) Ch. 19, 369-393 doi:https://doi.org/10.1515/9783110429985 (De Gruyter, 2018).

6 Tancrede-Bohin, E. *et al.* In vivo multiphoton imaging for non-invasive time course assessment of retinoids effects on human skin. *Skin Res Technol* **26**, 794-803, doi:https://doi.org/10.1111/srt.12877 (2020).

7 Pena, A. M. *et al. In vivo* melanin 3D quantification and z-epidermal distribution by multiphoton FLIM, phasor and Pseudo-FLIM analyses. *Scientific Reports* **12**, 1642, doi:https://doi.org/10.1038/s41598-021-03114-0 (2022).

8 Qiu, J. *et al.* The skin-depigmenting potential of Paeonia lactiflora root extract and paeoniflorin: In vitro evaluation using reconstructed pigmented human epidermis. *Int J Cosmet Sci* **38**, 444-451, doi:http://onlinelibrary.wiley.com/doi/10.1111/ics.12309/epdf (2016).