**In vivo** melanin 3D quantification and z-epidermal distribution by multiphoton FLIM, phasor and Pseudo-FLIM analyses

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ABSTRACT

Characterizing melanins *in situ* and determining their 3D z-epidermal distribution is paramount for understanding physiological/pathological processes of melanin neosynthesis, transfer, degradation or modulation with external UV exposure or cosmetic/pharmaceutical products.

Multiphoton fluorescence intensity- and lifetime-based approaches have been shown to afford melanin detection, but how can one quantify melanin *in vivo* in 3D from multiphoton fluorescence lifetime (FLIM) data, especially since FLIM imaging requires long image acquisition times not compatible with 3D imaging in a clinical setup? We propose an approach combining i) multiphoton FLIM, ii) fast image acquisition times, and iii) a melanin detection method, called Pseudo-FLIM, based on slope analysis of autofluorescence intensity decays from temporally binned data.

By comparison to FLIM bi-exponential and phasor analyses of synthetic melanin, melanocytes/keratinocytes coculture and *in vivo* human skin, we demonstrate that the fast decay shapes of melanin correspond to high slope values, a characteristic one can use to identify melanin *in vitro* and *in vivo*. We show that Pseudo-FLIM melanin detection is still feasible upon decreasing the image acquisition time, which opens the possibility to quantify melanin from 3D z-stacks. Using parameters of global 3D epidermal melanin density and z-epidermal distribution profile, we provide first insights into the *in vivo* knowledge of 3D melanin modulations with constitutive pigmentation versus ethnicity, with seasonality over 1 year and with topical application of retinoic acid or retinol. Applications of Pseudo-FLIM-based melanin detection encompass physiological, pathological, or environmental factors-induced pigmentation modulations up to whitening, anti-photoaging, or photoprotection products evaluation.

**Keywords:** Multiphoton, FLIM, Phasor, Pseudo-FLIM, melanin, 3D quantification, *in vivo* human skin, cells

1. INTRODUCTION

Characterizing melanins *in situ* and determining their 3D z-epidermal distribution is paramount for understanding physiological/pathological processes of melanin neosynthesis, transfer, degradation or modulation with external UV exposure or cosmetic/pharmaceutical products.

Currently, the gold standard method for melanin quantification is high-performance liquid chromatography (HPLC) chemical analysis of melanin degradation products. Although very specific, it requires *ex vivo* samples degradation and provides no information on melanin’s epidermal distribution. Fontana-Masson staining allows insights in 2D melanin distribution and content but only within living epidermis (e.g. 3-4).

Assessing melanin content and distribution in *in vivo*, in 3D, is still a challenging topic addressed over the years with different optical methods. Multiphoton fluorescence intensity- and lifetime-based approaches have been shown to afford melanin detection. Especially, multiphoton FLIM imaging of melanin samples such as synthetic, DOPA or Sepia melamins, skin and eye melanocytes, human hair and hair bulb, and human skin (e.g. 5-7, 9-11) indicate a specific bi-exponential decay behavior with a predominantly (>90% relative contribution) short-fluorescence lifetime component around ~100-200 ps and a mixed species phasor plot with short phase lifetime distribution. However, image acquisition time needed for
acquiring correct fluorescence decays for bi-exponential or phasor analysis is not compatible with 3D skin imaging in a clinical setup and in practice is limited to 2D imaging at selected epidermal depths.

In order to detect melanin from multiphoton FLIM-like data compatible with 3D in vivo acquisitions on human subjects, we propose to use an approach combining i) multiphoton FLIM, ii) fast image acquisition times, and iii) a melanin detection method, called Pseudo-FLIM, based on slope analysis of the autofluorescence intensity decay from temporally binned data (Figure 1).

![FLIM – Bi-exponential fit](image)

![FLIM - Phasor plot](image)

![Pseudo-FLIM analysis: Temporal binning → Ln of 2PEF Int. → Linear regression → Slope estimation](image)

Figure 1: Principle of FLIM bi-exponential, Phasor and Pseudo-FLIM analyses. a) Simulated mono-exponential (fluorophores A and B) and bi-exponential (mixed species of fluorophores A and B with respectively 90% and 10% relative contribution) two-photon excited fluorescence intensity decays (12.5 ns time range; 80 MHz); this shape of A&B bi-exponential decay can be measured in melanin containing samples. In FLIM bi-exponential analysis, the 2PEF intensity decay is adjusted with the function in a) to compute the values of short- \( \tau_1 \) and long-\( \tau_2 \) fluorescence lifetimes and their relative contributions \( a_1 \) and \( a_2 \). Images of FLIM bi-exponential fit and combination parameters such as amplitude- and intensity-weighted lifetimes are used for data analyses. b) FLIM phasor analysis transforms a decay into a phasor with polar coordinates \( g, s \), corresponding to the real and complex components of the Fourier transform, that can also be expressed as a function of modulation and \( \phi \) phase angle. Mono-exponential decays such as A and B will have their phasors on the semi-circle, whereas mixed species will have a phasor along a line connecting the two distinct lifetime phasors of A and B. The relative fractions \( f_A \) and \( f_B \) can be computed from the distances of A&B mixed species phasor to B and respectively A phasors. Images of \( g \) and \( s \) as well as combination parameters such as the apparent phase and modulation lifetimes and their relative fractions are used for data analyses. c) Pseudo-FLIM analysis firstly involves a temporal binning of the 2PEF decay into a reduced number of time channels with 2 ns integration time per channel (see c1, gray bars indicate the photon intensity of the mixed A&B species within the first 3-time channels). The 2PEF intensity of the binned first 3-time channels for simulated A, B and mixed A&B species is given in (c2). After a natural logarithm transformation (c3), a linear regression of the first 3-time channels is performed to calculate the slope of the decay which is multiplied by a -100 factor to create the Pseudo-FLIM slope parameter. The faster the decay, the higher the slope. Pseudo-FLIM image of the slope parameter is further processed for melanin detection by applying a threshold to keep the pixels with high slope values. Reprinted from Ana-Maria Pena et al. “In vivo melanin 3D quantification and z-epidermal distribution by multiphoton FLIM, phasor and Pseudo-FLIM analyses”, Scientific Reports 12(1), 1642 (2022), publisher Springer Nature, Copyright © 2022, The Author(s)13.
2. RESULTS AND CONCLUSIONS

The full results of this work can be found in Pena et al. Scientific Reports 12(1), 1642 (2022) 13. Hereafter, we only provide a brief description.

We compared Pseudo-FLIM to FLIM bi-exponential and phasor analyses of synthetic melanin, melanocytes and keratinocytes coculture and in vivo human skin (Figure 2; full results in Pena et al. 13) and demonstrated that the fast decay shapes of melanin containing samples correspond to high slope values, a characteristic one can use to identify melanin containing pixels in vitro or in vivo. In human skin, melanin pixels characterized by a short-fluorescence lifetime $\tau_1<100$ ps, a relative contribution $a_1[\%]>90\%$ and phasor parameters $g>0.5$ & $s<0.3$ are detected by Pseudo-FLIM analysis, which also includes other pixels with phase lifetimes $\tau_\phi<1.1$ ns.

![Figure 2: Comparison of FLIM bi-exponential fitting, Phasor and Pseudo-FLIM methods for melanin detection in vivo on human skin. Multiphoton 2D 2PEF FLIM images acquired at different depths from the skin surface to the dermis, within stratum corneum (SC) disjunctum, corneum compactum, granulosum (SG), spinosum (SS), basale (SB) and superficial dermis were analyses with the three methods. (top) FLIM bi-exponential fitting analysis images of the short $\tau_1$ fluorescence lifetime parameter. (middle) Phasor plots (s versus g scatters) of the different skin layers, color coded using the color scale of $\tau_1$ and respectively $\tau_2$ parameters, along with the corresponding phase lifetime image. (bottom) Pseudo-FLIM analysis images of the slope parameter highlighting pixels with a fast decay. The arrows indicate the fast decay pixels within the blood capillary. Reprinted from Ana-Maria Pena et al. “In vivo melanin 3D quantification and z-epidermal distribution by multiphoton FLIM, phasor and Pseudo-FLIM analyses”, Scientific Reports 12(1), 1642 (2022),” publisher Springer Nature, Copyright © 2022, The Author(s)13.](image)

We also demonstrated that Pseudo-FLIM melanin detection is still feasible upon decreasing the image acquisition time, which opened the possibility to quantify melanin from 3D z-stacks (Figure 3). Using parameters of global 3D epidermal melanin density and z-epidermal distribution profile, we provide first insights into the in vivo knowledge of 3D melanin modulations with constitutive pigmentation versus ethnicity (Figure 4). Despite the small investigated epidermal volume,
Multiphoton global 3D epidermal melanin density was found to be highly correlated with skin color ITA value (Figure 4), in agreement with ex vivo histology and HPLC chemical analysis data.

Figure 3: In vivo 3D multiphoton images of human skin – acquisition of combined 2PEF-FLIM (4 time channels)/SHG z-stacks compatible with Pseudo-FLIM melanin detection. 2PEF intensity is shown in cyan hot color, SHG in red and Pseudo-FLIM melanin mask pixels in purple. High 2PEF signal intensities appear in white color. Images are extracted from a z-stack of 70 en face images acquired 2,346 µm z-step. a) stratum corneum disjunctum; b) stratum corneum disjunctum / compactum interface; c-d) compactum / granulosum interface; e-f) stratum granulosum; g-h) stratum spinosum; i) stratum basale; j-o) stratum basale/dermis interface; p) superficial dermis. Within the blood capillaries, Pseudo-FLIM detects cells with high slope values, fast decays, that most probably emit 2PEF signals from hemoglobin (see arrows). As the image acquisition time is slower than the blood flow, some blood cells appear with a deformed shape as indicated by the arrow in image m. Reprinted from Ana-Maria Pena et al. “In vivo melanin 3D quantification and z-epidermal distribution by multiphoton FLIM, phasor and Pseudo-FLIM analyses”, Scientific Reports 12(1), 1642 (2022),” publisher Springer Nature, Copyright © 2022, The Author(s).
Figure 4: Modulation of global epidermal 3D melanin density and z-epidermal distribution with skin color ITA grade and skin ethnicity in constitutive pigmentation study. a) Raw 2PEF intensity images (cyan hot) and Pseudo-FLIM melanin

- **PIDERMIS**
- **MELANIN**
- **3D DENSITY [%]**
  - 50
  - 30
  - 20
  - 10
  - 40

- **Skin Color - ITA grade**
  - I
  - II
  - III
  - IV
  - V

- **Skin colour group**
  - I - very light
  - II - light
  - III - intermediate
  - IV - tanned
  - V - brown

- **3D MELANIN DENSITY Z-PROFILE [%]**
  - 75
  - 50
  - 25
  - 0

- **CORRELATION MATRIX**

|       | L     | a     | b     | ITA   |
|-------|-------|-------|-------|-------|
| L     | -0.89 | -0.64 | 0.99  |       |
| a     | -0.89 | 0.78  | -0.89 |       |
| b     | -0.64 | 0.78  | -0.70 |       |
| ITA   | 0.99  | -0.89 | -0.70 |       |
| ED    | 0.87  | 0.79  | 0.62  | -0.86 |
| SC    | -0.56 | 0.50  | 0.32  | -0.55 |
| SC Disj | -0.41 | 0.31  | 0.09  | -0.40 |
| SC Comp | -0.46 | 0.48  | 0.43  | -0.45 |
| LED   | -0.89 | 0.80  | 0.63  | -0.89 |
| SGranu | -0.60 | 0.51  | 0.41  | -0.59 |
| SSpin | -0.90 | 0.76  | 0.56  | -0.89 |
| SBas  | -0.86 | 0.82  | 0.67  | -0.85 |

- **Skin Ethnicity**
  - African origin
  - Asiatic origin
  - European origin

- **Pearson**
  - 1.0
  - 0.5
  - 0.0
  - -0.5
  - -1.0

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masks (purple) acquired within the basal layer of human forearm skin. High signal intensities appear in white color. b) Modulation of 3D epidermal melanin density with ITA group and c) with both ITA group and skin ethnicity. The data are expressed as boxplots with fences. d) Correlation matrix of the skin colorimetric parameters and multiphoton melanin density estimated in the epidermis and its sub-layers. The data correspond to the Pearson correlation coefficients. An absolute value of the Pearson correlation coefficients between [0.6, 0.8] and [0.8, 1.0] indicate a strong and respectively a very strong correlation.

Malignant melanin z-epidermal distribution profiles (mean 3D melanin density estimated in 12 thickness-normalized epidermal layers from 1—DEJ level to 12—SC level) of e) different skin color ITA groups and f) different skin ethnicity. The insert in f) is showing the z-profiles of different skin color ITA groups for Asiatic and European origins. The z-profiles data are expressed as mean ± confidence intervals of the mean. ED (global epidermis), SC - stratum corneum, SC Disj - stratum corneum disjunctum, SC Comp - stratum corneum compactum, LED – living epidermis, SGranu - stratum granulosum, SSpin – stratum spinosum and SBas - stratum basale. Reprinted from Ana-Maria Pena et al. “In vivo melanin 3D quantification and z-epidermal distribution by multiphoton FLIM, phasor and Pseudo-FLIM analyses”, Scientific Reports 12(1), 1642 (2022), publisher Springer Nature, Copyright © 2022, The Author(s).13

Moreover, our results (see details in Pena et al.13) indicate that multiphoton imaging can provide quantitative data of interest in studying pigmentation modulations under different conditions (constitutive and acquired pigmentation, aging14, natural UV exposure15 or application of topical retinoids15, 16 or corticosteroids16), but its ability to accurately assess melanin heterogeneity in skin pigmentation disorders remains to be studied.

Multiphoton Pseudo-FLIM based melanin detection method could also bring new insights into the knowledge of some underlying biological mechanisms of pigmentation modulations appearing through either redistribution of existing melanin and/or de novo melanin synthesis. It will certainly help understating the mechanisms of skin photobiology (how melanin is modulated with different UV wavelengths and doses) and it will also contribute to developing tomorrow’s photoprotection products.

More generally, the in vitro and in vivo applications of multiphoton Pseudo-FLIM based melanin detection and quantification of its global 3D density and z-epidermal distribution encompass physiological, pathological, or environmental factors-induced pigmentation modulations up to whitening, anti-photoaging, or photoprotection products evaluation.

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