Two-photon excited fluorescence spectroscopy and imaging of melanin *in-vitro* and *in-vivo*

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ABSTRACT

The ability to detect early melanoma non-invasively would improve clinical outcome and reduce mortality. Recent advances in two-photon excited fluorescence (TPEF) *in vivo* microscopy offer a powerful tool in early malignant melanoma diagnostics.

The goal of this work was to develop a TPEF optical index for measuring relative concentrations of eumelanin and pheomelanin since *ex vivo* studies show that changes in this ratio have been associated with malignant transformation. We acquired TPEF emission spectra (λ_{ex}=1000 nm) of melanin from several specimens, including human hair, malignant melanoma cell lines, and normal melanocytes and keratinocytes in different skin layers (epidermis, papillary dermis) in five healthy volunteers *in vivo*. We found that the pheomelanin emission peaks at around 620 nm and is blue-shifted from the eumelanin with broad maximum at 640-680nm.

We defined "optical melanin index" (OMI) as a ratio of fluorescence signal intensities measured at 645 nm and 615nm. The measured OMI for a melanoma cell line MNT-1 was 1.6±0.2. The MNT-46 and MNT-62 lines (Mc1R gene knock-down) showed an anticipated change in melanins production ratio and had OMI of 0.55±0.05 and 0.17±0.02, respectively, which strongly correlated with HPLC data obtained for these lines. Average OMI measured for basal cells layers (melanocytes and keratinocytes) in normal human skin type I, II-III (not tanned and tanned) *in vivo* was 0.5, 1.05 and 1.16 respectively. We could not dependably detect the presence of pheomelanin in highly pigmented skin type V-VI. These data suggest that a non-invasive TPEF index could potentially be used for rapid melanin ratio characterization both *in vitro* and *in vivo,* including pigmented lesions.

Keywords: TPEF spectroscopy, eumelanin, pheomelanin, human skin

1. INTRODUCTION

Human skin and hair color is determined by melanin type and content. There are two major forms of it: eumelanin and pheomelanin. Eumelanin is brown to black in color while pheomelanin is yellow to red. The regulation of the production of eumelanin versus pheomelanin involves the interaction of the melanocortin 1 receptor (Mc1R) on the surface of the melanocyte with melanocyte stimulating hormone (MSH) or with the agouti signaling protein. The binding of MSH to Mc1R results in the formation of eumelanin while the binding of the agouti protein to Mc1R leads to the production of pheomelanin.

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Epidemiology studies have shown that red hair and light skin color are risk factors for melanoma development [1, 2]. Mutations in human Mc1R gene is associated with red hair and light skin color, which is characterized by a high pheomelanin/eumelanin ratio [3]. While eumelanin can serve as anti-oxidant to scavenge free radicals [4], pheomelanin can become photosensitizer and generates reactive oxygen species after UV radiation [5, 6].

Currently, total melanin measurement in biological specimens is often achieved by measuring light absorption at 450-475 nm after alkaline degradation [7]. Due to a lack of understanding of the structures of eumelanin and pheomelanin, it has not been possible to directly measure these two types of melanins. Ito and Fujita developed HPLC-based method to indirectly measure eumelanin and pheomelanin after a series of degradation and oxidation steps [8, 9]. Because of the complicated processing steps and amplification constant used in this method, a small error in the HPLC measurement could result in a large difference in the final reading, hence the results are difficult to repeat.

Thus, rapid, non-degrading, non-invasive optical methods for melanin ratio measurements would be highly desirable. However, up to date only a pump-probe imaging approach [10, 11] made qualitative imaging of pheomelanin and eumelanin possible and was demonstrated in excised human pigmented lesions and *in vivo* in murine model. Other optical approaches rely on reflectance spectroscopy [12] and absorption spectroscopy [13].

There is a limited amount of data in literature concerning fluorescence properties of naturally occurring melanins. Excitation of fluorescence in visible range was deemed impractical due to low quantum yield, efficient absorption in a wide range causing self-quenching of emission, photodamage including burning, etc. Near infrared (NIR) 2-photon excitation makes the method more attractive due to a lower scattering in turbid media, limited one-photon absorption and thus lower photodamage. First results for a bulk melanin emission properties *in vivo* in human skin were reported for "conventional" two-photon and stepwise two-photon excitation at 810 nm in [14].

The goal of this work was to elicit the fluorescence properties of naturally occurring eumelanin and pheomelanin and to develop a non-invasive TPEF optical index for determining relative concentrations of the two compounds.

2. METHODS

2.1. Cell and raft cultures.

MNT-1 cells are established from metastatic melanoma, which appear to be dark in culture. MNT-46 and MNT-62 are two clones derived from MNT-1 transfected Mc1R shRNA and selected with puromycin.
Normal human fibroblasts and normal kertinocytes were harvested from neonatal foreskin as previously described [15]. Fibroblasts, MNT1, MNT46 and MNT62 were maintained in Advanced DMEM supplemented with 2% Fetal Bovine Serum and 2mM Glutmax. All routine culture medium and supplements are from Life Technology. Kertinocytes were maintained in KGM2 (Lonza). Raft cultures were prepared as previously described with slight modification. Rafts established for the current experiments have fibroblasts at density of 100,000 cells per raft and kertinocytes at a density of 500, 000 per raft. MNT cells were mixed with kertinocytes at a ratio of 1 to 10 before plating on the established dermis. At air liquid interface, rafts were maintained in 1:1 of supplemented Advanced DMEM and KGM2 on top of a 25 mm diameter 8 micron Polycarbonate membrane (GE).

2.2. Eumelanin and pheomelanin measurement by HPLC.

PTCA (Pyrrole-2, 3, 5-tricarboxylic acid) and 4-AHP (4-Amino-3-hydroxyphenylalanine) were used as markers for eu- and pheo- melanin content respectively and the ratio of PTCA to AHP served as the ratio of eu- to pheo- melanin content. Eumelanin and pheomelanin samples were treated and analyzed as described by Ito and Wakamatsu [16, 17] with slight modification. In brief, eumelanin sample was oxidized with H_2O_2 under basic condition [17] while pheomelanin structure was destroyed under reduced acidic condition using HI and H_3PO_2 [16]. Pheomelanin lysate was further purified via a SCX column (Hypersep SPE 500mg/3mL SCX Hypersil column, thermo scientific, wash with 3mL water and methanol, elute with 1mL 5M NH_4Ac) before analysis.

Eumelanin separation was performed on Waters e2695 system with 2478 UV detector at 269nm using a C18 column (Atlantis T3, 4.6 x 250 mm, 5 μm particle size). PTCA was determined in isocratic mode with a mobile phase of 18%

methanol in 20mM potassium phosphate buffer (pH 2.12) at a flow rate of 0.7 ml/min under room temperature (Injection volume: 80 µL); HPLC condition for pheomelanin: Samples were analyzed with an HPLC system consisting of Agilent 1100 series instrument, a C18 column (Atlantis T3, 4.6 x 250 mm, 5 µm particle size), an Electro-chemical detector (Agilent 35900E interface and LC-4C Amperomrtric Detector), a reference electrode: Ag/AgCl and a Working electrode: Pt. 4-AHP was eluted at a flow rate of 0.5ml/min in gradient mode: 0-30min, from 100%A to 100%B, 30-40min, 100%B, equilibrate column with 100%A for 15min before next run. Mobile phase A: 0.1% acetic acid in 5% methanol with 1.5mM Octanesulfonic acid sodium salt and 0.1 mM EDTA (pH4.0); Mobile phase B: 0.1% acetic acid in 90% methanol with 1.5mM Octanesulfonic acid sodium salt and 0.1 mM EDTA (pH4.0). The working voltage was set to +700 mV.

2.4. Two-photon imaging and spectroscopy system.

Two-photon excited fluorescence emission spectra of melanins in-vitro were obtained using 32-channel Meta detector of Zeiss LSM 510 Meta NLO microscopy system. Excitation wavelength used was 1000 nm from Chameleon-Ultra femtosecond pulsed tunable (from 690 nm to 1040 nm) laser (Coherent Inc.). All spectra were acquired at the identical settings (excitation power, detector settings) and normalized to unity at 560 nm. Ratios of signal intensity at 645 nm (for eumelanin) to 615 nm (pheomelanin) were measured and averaged for 20-25 individual cells for each MNT-1, MNT-46, and MNT-64 cell lines.

In vivo measurements were performed with the same system equipped with the objective inverter (LSM Tech. Inc, Stewartstown, PA). This innovative device is a unique tool for intra-vital microscopy. It converts any inverted microscope into an upright by simultaneously changing the orientation of the objective and moving the sample off of the stage. This allows maximum flexibility in sample positioning and handling while still retaining the ability to automatically acquire z-stacks. All *in vivo* measurements were conducted according to an approved institutional protocol, and with informed consent by all participants.

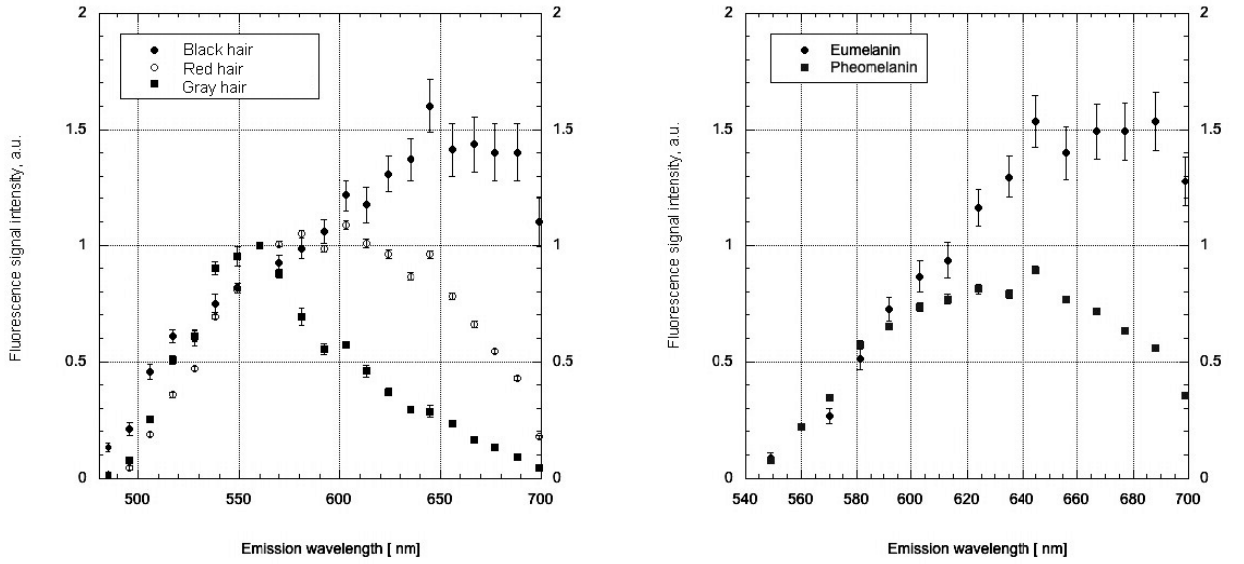
Objectives used were Zeiss EC Plan-Neofluar 40x (N.A.=1.3 oil immersion) for spectral measurements of melanin in hair and Zeiss Achroplan (40x N.A.=0.8, water immersion) for all cellular rafts and *in vivo* measurements.

3. RESULTS

As a source of naturally occurring eumelanin and pheomelanin in humans we used human hairs of different colors. Pigments are produced by melanocytes located in hair follicles in dermis and deposited in a melanosomes, which then distributed along the hair shaft. The pigment in blond and red hair contains predominantly pheomelanin, while dark brown and black color is due to eumelanin. Gray hair is devoid of any pigment [18].

All spectra were normalized to unity at 560 nm (Fig.1 a). The fluorescence emission spectrum of a colorless gray hair represented the sum of all of native cellular and extracellular fluorescence not associated with melanin and was subtracted from the spectra of pigmented samples. The remaining portion was attributed to a fluorescence emission of eumelanin (black hair) and pheomelanin (red hair), respectively (Fig.1 b).

Pheomelanin fluorescence emission peaked around 620 nm. Eumelanin displayed red-shifted compared to pheomelanin broad fluorescence emission peak at 640-680 nm. Samples that mainly contained red pigment didn't show any signs of damage after scanning with both 900 nm and 1000 nm. Samples with significant content of eumelanin (all dark brown and black samples) showed a very high susceptibility to photodamage by a scanning laser (burns) at a much lower incident power that is most probably due to a one-photon absorption of NIR light by eumelanin. Excitation at 1000 nm was deemed more appropriate due to less absorbance and hence less damage to dark samples while peak emission intensity was comparable to that obtained at 900 nm. Excitation wavelengths longer that 1000 nm weren't practical because of a sharp drop in a laser power output and light transmission properties of used microscopy objectives.



a b

Fig.1. Fluorescence emission spectra of red (open circle), black (filled circle) and gray (squares) human hair (a). Excitation wavelength was 1000 nm. Fluorescence emission spectra of pheomelanin (circles, red hair) and eumelanin (squares, black hair) after autofluorescence (gray hair) subtraction (b).

In order to ascertain the usability of spectral measurements toward development of optical melanin index (OMI), which is defined as a ratio of fluorescence signal intensities measured at 645 nm (eumelanin) and 615nm (pheomelanin), we compared the results obtained by chemical (HPLC) and optical measurements. We used a set of three human melanoma–derived cell lines (MNT-1, MNT-46 and MNT-62). MNT-1 cells are established from human metastatic melanoma. MNT-46 and MNT-62 are two Mc1R gene knock-down clones derived from MNT-1. Both clones showed about 70-80% of reduced protein accumulation of Mc1R as examined by western blot (data now shown) and suggest a drastic change in eumelanin/pheomelanin ratio [19, 20].

For optical measurements MNT cells were co-cultured with human keratinocytes and seeded in 3-D collagen scaffolds to maintain melanin production. The rafts were transferred to 35-mm imaging Petri dishes with coverslip #1 on the bottom immediately before the imaging. Rafts were "flipped" to place cells directly onto the coverslip. The spectra were obtained from 20-30 pigmented cells for each cell line. Spectra were corrected by subtracting the autofluorescence from the non-pigmented cells present in the each sample. Fig.2 demonstrates a good correlation between the eumelanin to pheomelanin ratios measured by HPLC and fluorescence emission analysis.

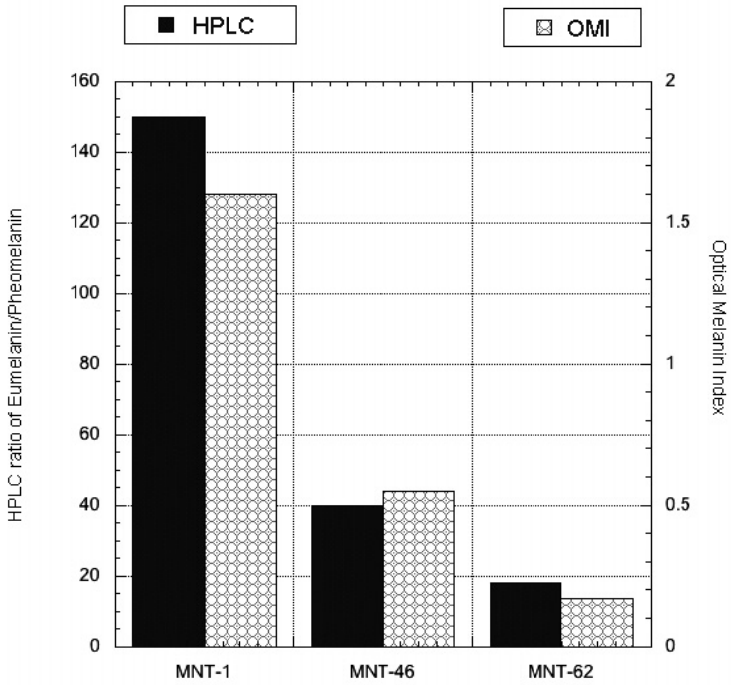


Fig. 2. Comparison of ratios of eumelanin to pheomelanin measured with HPLC-based chemical (left axis) and optical fluorescence emission analysis (right axis).

Microscopy imaging and measurements of melanin spectra in human skin *in vivo* were performed on the same Zeiss LSM510 microscope equipped with the objective inverter. Imaging dish, fixed to a post by a holder made in-house, provided both a way to hold an immersion medium (water) in front of an objective and offered an extra stabilization for the sample (skin of a human arm) (Fig.3).

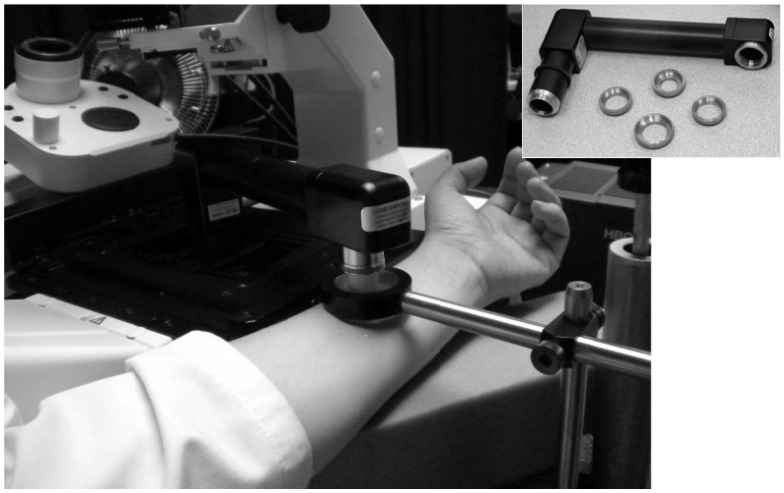


Fig. 3. Microscopy set-up for *in-vivo* TPEF imaging and spectroscopy measurements. Insert (top right) shows the objective inverter used.

Spectra were acquired at the epidermal-dermal junction and in epidermis below the stratum corneum (Fig.4). Epidermal-dermal junction provided the most reliable data because the basal cellular layer containing pigmented cells was easily discernable as surrounding dermal papilla [21] .

OMI were measured in normal human skin *in vivo* by the same protocol as was used for cells *in vitro*, including subtraction of autofluorescence of non-pigmented cells. Average OMI for basal cells layers (melanocytes and keratinocytes) in skin type I, II-III (both not tanned and slightly tanned) were 0.5, 1.05 and 1.16 respectively. We could not reach epidermal-dermal junction in highly pigmented skin type V-VI or dependably detect the presence of pheomelanin in more superficial layers of epidermis in those darkly pigmented skin.

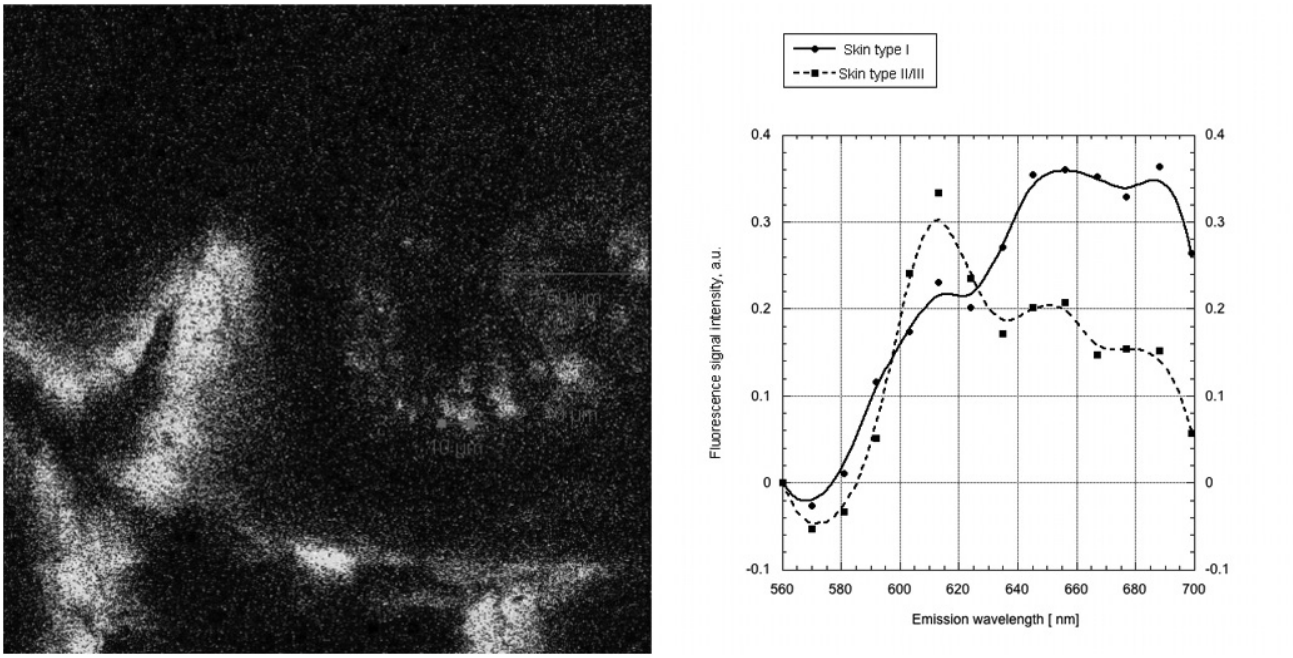


Fig. 4. *In vivo* spectral image of epidermal-dermal junction in skin type II/III. Typical spectra of pheomelanin-rich (skin type I-dotted line) and eumelanin-rich (tanned skin type II/III-continuous line) pigmented cells are similar to those of recorded in hair.

However, this should not discourage the development of optical methods for characterization of normal skin and pigmented lesions partially due to a fact that lighter skin pigmentation represents a major risk factor for developing UV-induced skin diseases.

CONCLUSIONS

We report here two-photon excited fluorescence spectra of two forms of melanin: eumelanin and pheomelanin, naturally occurring in human skin. In order to ascertain the usability of spectral measurements toward development of optical melanin index (OMI) we used a set of three human melanoma–derived cell lines with changing levels of melanins in which the ratios of eumelanin to pheomelanin were quantified by chemical method and fluorescence emission analysis. OMI shows a good correlation with HPLC-derived data. Spectral measurements of lighter human skin types (I-III) *in vivo* showed expected increase in measured OMI values.

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REFERENCES

1. Sturm, R.A., *Skin colour and skin cancer -MC1R, the genetic link.[Review].* Melanoma Research, 2002. **12**(5): p. 11.
2. Gudbjartsson, D.F., et al., *ASIP and TYR pigmentation variants associate with cutaneous melanoma and basal cell carcinoma.* Nat Genet, 2008. **40**(7): p. 886-891.
3. Naysmith, L., et al., *Quantitative Measures of the Effect of the Melanocortin 1 Receptor on Human Pigmentary Status1.* J Investig Dermatol, 2004. **122**(2): p. 423-428.
4. Sarna, T., et al., *Interaction of radicals from water radiolysis with melanin.* Biochimica et Biophysica Acta (BBA) - General Subjects, 1986. **883**(1): p. 162-167.
5. Jimbow, K., *Current Update and Trends in Melanin Pigmentation and Melanin Biology.* The Keio Journal of Medicine, 1995. **44**(1): p. 9.
6. Hill, H.Z. and G.J. Hill, *UVA, Pheomelanin and the Carcinogenesis of Melanoma.* Pigment Cell Research, 2000. **13**: p. 140-144.
7. Kadekaro, A.L., et al., *Cutaneous Photobiology. The Melanocyte vs. the Sun: Who Will Win the Final Round?* Pigment Cell Research, 2003. **16**(5): p. 434-447.
8. Ito S, F.K., *Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatograph.* Anal Biochem, 1985. **144**(2): p. 9.
9. Ito S, W.K., *An improved modification of permanganate oxidation of eumelanin that gives a constant yield of pyrrole-2,3,5-tricarboxylic acid.* Pigment Cell Res., 1994. **7**(3): p. 4.
10. Matthews, T.E., et al., *Pump-Probe Imaging Differentiates Melanoma from Melanocytic Nevi.* Science Translational Medicine, 2011. **3**(71): p. 71ra15.
11. Matthews, T.E., et al., *In vivo and ex vivo epi-mode pump-probe imaging of melanin and microvasculature.* Biomed. Opt. Express, 2011. **2**(6): p. 1576-1583.
12. Marchesini, R., A. Bono, and M. Carrara, *In vivo characterization of melanin in melanocytic lesions: spectroscopic study on 1671 pigmented skin lesions.* Journal of Biomedical Optics, 2009. **14**(1): p. 014027.
13. Zonios, G., Aikaterini Dimou, Ioannis Bassukas, Dimitrios Galaris, Argyrios Tsolakidis and Efthimios Kaxiras, *Melanin absorption spectroscopy: new method for noninvasive skin investigation and melanoma detection.* Journal of Biomedical Optics, 2008. **13**.
14. Scholz, et al., *Uncovering of melanin fluorescence in human skin tissue*. Biophotonics 2007: Optics in Life Science, ed. G.v.B. Ju□rgen Popp. Vol. 6633 2007, Belligham, ETATS-UNIS: Society of Photo-Optical Instrumentation Engineers. 7.
15. Chiu, L.L., et al., *Photodynamic therapy on keloid fibroblasts in tissue-engineered keratinocyte-fibroblast co-culture.* Lasers in Surgery and Medicine, 2005. **37**(3): p. 231-244.
16. Wakamatsu, K. and S. Ito, *Advanced Chemical Methods in Melanin Determination.* Pigment Cell Research, 2002. **15**(3): p. 174-183.
17. Ito, S. and K. Wakamatsu, *Chemical Degradation of Melanins: Application to Identification of Dopamine-melanin.* Pigment Cell Research, 1998. **11**(2): p. 120-126.
18. Haywood, R.M., M. Lee, and C. Andrady, *Comparable Photoreactivity of Hair Melanosomes, Eu- and Pheomelanins at Low Concentrations: Low Melanin a Risk Factor for UVA Damage and Melanoma?†.* Photochemistry and Photobiology, 2008. **84**(3): p. 572-581.
19. Scott, M.C., et al., *Human melanocortin 1 receptor variants, receptor function and melanocyte response to UV radiation.* Journal of Cell Science, 2002. **115**(11): p. 2349-2355.
20. Abdel-Malek, Z.A., et al., *The Melanocortin 1 Receptor and the UV Response of Human Melanocytes—A Shift in Paradigm†.* Photochemistry and Photobiology, 2008. **84**(2): p. 501-508.
21. Dimitrow, E., et al., *Sensitivity and Specificity of Multiphoton Laser Tomography for In Vivo and Ex Vivo Diagnosis of Malignant Melanoma.* J Invest Dermatol, 2009. **129**(7): p. 1752-1758.