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Skin collagen can be accurately quantified through noninvasive optical method: Validation on a swine study

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Abstract

Background/purpose: Diffuse reflectance spectroscopy (DRS) is a noninvasive optical technology characterized by relatively low system cost and high efficiency. In our previous study, we quantified the relative concentration of collagen for the individual keloid patient. However, no actual value of collagen concentration can prove the reliability of collagen detection by our DRS system.

Methods: Skin-mimicking phantoms were prepared using different collagen and coffee concentrations, and their chromophore concentrations were quantified using the DRS system to analyze the influence of collagen and other chromophores. Moreover, we used the animal study to compare the DRS system with the collagen evaluation of biopsy section by second-harmonic generation (SHG) microscopy at four different skin parts.

Results: In the phantom study, the result showed that coffee chromophore did not severely interfere with collagen concentration recovery. In the animal study, a positive correlation (r=.902) between the DRS system and collagen evaluation with SHG microscopy was found.

Conclusions: We have demonstrated that the DRS system can quantify the actual values of collagen concentration and excluded the interference of other chromophores in skin-mimicking phantoms. Furthermore, a high positive correlation was found in the animal study with SHG microscopy. We consider that the DRS is a potential technique and can evaluate skin condition objectively.

KEYWORDS

collagen concentration, collagen phantom, diffuse reflectance spectroscopy, Lanyu pig, noninvasive measurement, skin collagen

1 | INTRODUCTION

Type 1 collagen not only is the most abundant protein in the dermis layer but is responsible for skin strength and elasticity.[1] Therefore, wrinkle formation has been implicated in the decrease in collagen concentration in the skin.[2] Collagen concentration is often affected by skin aging (intrinsic),[3] photo-damage (extrinsic), wound healing process,[4] and skin disease, particularly keloid and scleroderma.[5-7] Traditionally, collagen is categorized differently from melanin or hemoglobin, which are pigments that can be evaluated by visual colors.[8] Dermal collagen content has been evaluated mostly by histological examination of previously excised skin specimens. To quantitatively evaluate collagen concentration over time, a tool that allows in vivo assessment of changes in collage content is necessary. Multi-harmonic generation (MHG) microscopy and diffuse reflectance spectroscopy (DRS) are two novel technologies used for noninvasive assessment of collagen content changes in the skin.[6,9-11] MHG combined with second-harmonic generation (SHG) and third-harmonic generation imaging methods can assess dermal morphological changes at a penetration depth close to 300 μm

and can clearly observe the junction between the epidermis and dermis.[12] However, the average thickness of the facial skin is at least 0.8 mm.[13] MHG microscopy is too precise to obtain the entire skin collagen concentration information. DRS can characterize the optical properties (absorption and scattering properties, μ_a, μ_s') of skin tissue in vivo at low system cost and high measurement efficiency.[6,14] Moreover, the absorption spectra derived from the DRS measurements can be translated into the concentrations of skin tissue chromophores, such as collagen, hemoglobin, melanin, and water.[11,14] Previously, we performed a study using our system to measure the optical properties and collagen concentration of keloid scars. We demonstrated that the DRS system not only can quantify the relative collagen concentration, water content, and oxygen saturation but can also differentiate keloid lesions with differing severities; furthermore, water content and oxygen saturation can be used to monitor the therapeutic response of keloid.[11]

In the present study, we measured skin-mimicking phantoms with different collagen and coffee concentrations and analyzed their chromophore concentrations. We also performed an animal experiment with Lanyu pig using the DRS system and conducted biopsy on skin tissues for SHG microscopy to quantitatively analyze collagen fibrillar structure. We then proposed a collagen qualitative technology focusing on the potential evaluation of the study for in vivo clinical application.

2 | MATERIALS AND METHODS

2.1 | DRS system and quantification of chromophore concentration

A noninvasive approach was developed to evaluate collagen concentration on the basis of the measurement of diffuse reflectance from keloid scars and normal skin.[6,11] The DRS system used in the present study and the picture of collagen phantoms are illustrated in Figure 1. Briefly, a fiber optical probe comprising three multimode fibers arranged in line with 0.22 numerical aperture and 400 μm core diameter was used for light delivery and collection. The distances of the two source fibers from the detection fiber were 1 and 2 mm. The light source was a supercontinuum laser (SuperK COMPACT, NKT Photonics, Denmark), which could illuminate a 450-2400 nm wavelength region spectrum. An optical switch (Piezosystem, Jena, Germany) was used to switch the laser to 1 or 2 mm source fiber for the measurement of phantoms and injection of light into the skin tissue. The detection fiber receiving the reflectance spectra was connected to a spectrometer (QE65000; Ocean Optics, Dunedin, FL, USA). All the system controls and data collection are performed on a laptop installed with the MATLAB® software (MathWorks, Natick, MA, USA). The average time required for a single complete measurement was less than 1 s.

The algorithm employed to evaluate the collagen concentration of objects from the diffuse reflectance was described elsewhere.[15] Briefly, the inverse model used for determining optical properties of the diffuse reflectance was an artificial neural network trained by Monte Carlo simulation results. The recovered absorption spectra were fit to known absorption spectra of the main chromophores to extract the tissue or phantom chromophore concentrations.[6,11,14] The known chromophore absorption spectra included instant coffee, collagen, gelatin base, and water in the collagen phantom study and oxygenated hemoglobin, deoxygenated hemoglobin, melanin, water, and collagen in the Lanyu pig study.

2.2 | Skin-mimicking phantoms with different collagen and coffee concentrations

The optical properties of skin-mimicking phantoms can be stable for at least 2 weeks in biomedical optical research because of their satisfactory temporal stability.[16] In this study, the phantoms contained edible gelatin with deionized water, glycerin, and TiO_2 as base as well as collagen and coffee powder (Rich Blend, NESCAFE, Taiwan). Collagen powder was derived from raw swine skin, however, the collagen powder was not ultrapure and contained about 70% of collagen since the type 1 collagen constitutes only 70% of the dry weight of the skin.[17] The absorption spectrum of instant coffee has highly similar characteristics with that of human melanin. Both chromophores do not possess distinct spectral features but exhibit a power law-like decay from

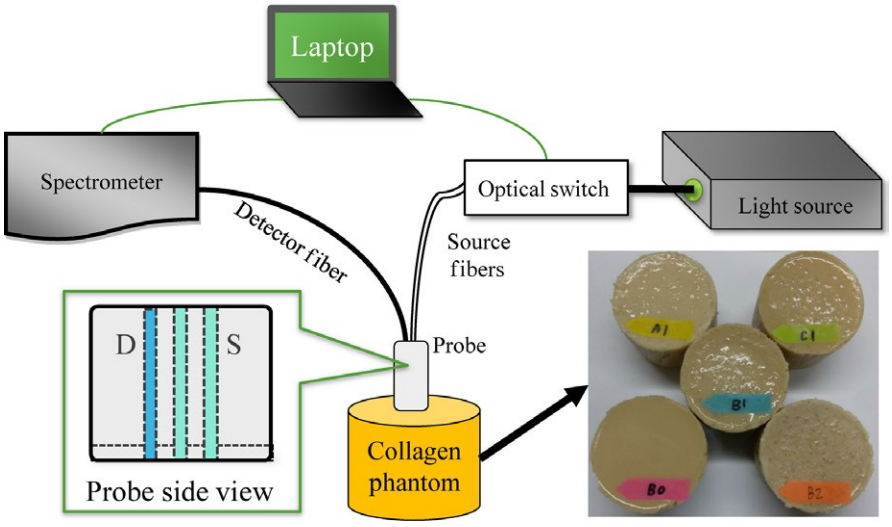

FIGURE 1 The configuration of the DRS system and photo of collagen phantoms. D: detector fiber; S: source fibers [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Recipe of skin-mimicking phantom study

	A1	B0	B1	B2	C1
Collagen (mg)	2	0	2	10	2
Coffee (mg)	300	500	500	500	700
Gelatin (W/V)			10%		
TiO_2 (mg)			470		
Glycerin (mL)			60		
Water (mL)			140		

ultraviolet to near infrared.[18] Table 1 describes the specifications of each skin-mimicking phantom. All the basic materials were well prepared at one time and were added with different concentrations of coffee and collagen powder separately to minimize the artificial error.

At the beginning, the deionized water was heated to 60°C and gelatin powder was slowly added to the water. The TiO_2 was also added to the glycerin in a gentle manner. The mixture was either strongly stirred during the process to obtain well-graded aggregate or distributed. Next, two mixtures were combined to form the solution base and were placed into the ultrasonic cleaner. The mixtures were then cooled to 50°C for 3 h to homogenize them and reduce the bubbles produced by the previous stirring process. Furthermore, the solution base was separated into five molds and allowed to cool down to 40°C. The desired amounts of collagen and coffee powder were added slowly with gentle stirring. Finally, when all the molds had been well stirred, and the entrapped air on the superficial of phantoms was removed, the molds were placed into a 4°C refrigerator for 10 h to allow the phantoms to fully cross-link.

2.3 | Lanyu pig experiment for skin collagen concentration evaluation and comparison with SHG microscopy

Lanyu pig is a small-ear miniature pig and frequently used as a model for human wound healing because of the relative thickness of its epidermis and dermis is similar to those of the human skin.[19,20] In this study, we selected a 4-month-old male Lanyu pig as the animal model. The pig's hair was shaved 1 day before the experiment to reduce skin irritation. The selected four measurement skin parts are shown in Figure 2. Sites A, B, and C are arranged in rows and perpendicular to the trunk from the abdominal side to dorsal side, and site D is close to the pelvic hip. At the beginning of the experiment, the pig was anesthetized with an intravenous injection of Zoletil 50 (2.2 mg/kg), and then nitrous oxide and halothane were administered through inhalation for the maintenance of general anesthesia. Each black square in Figure 2 has 3×3 cm^2, indicating the DRS measurement sites. After the measurement, approximately 1×1 cm^2 of skin sample in the center was cut for SHG measurement. These wounds had been treated and dressed appropriately. The protocol had been reviewed and approved by the Institutional Animal Care and Use Committee (no. 102284) in the National Cheng Kung University (NCKU) Hospital. The skin samples were washed with PBS and then fixed in 10% formalin solution for over 36 h. After fixation, the samples were dehydrated and embedded in paraffin. Each skin sample paraffin was sliced into 6 μm thick sections, and eight sections, which were close to the sample center, were selected and stained using hematoxylin and eosin (H&E) stain for SHG microscopy measurement.

All the SHG microscopy measurements of skin sections were performed by the Center for Micro/Nano Science and Technology in NCKU, which is one of the key institutes for nanotechnology and research in Taiwan. The lab-made two-photon microscope used in this study was similar to that used by the previous study of Lin et al.[21] Each scanning piece is 200×200 μm^2. A single section had 6×13 pieces, and each boundary between any two neighboring pieces was overlapped by 14 pixels. The intensity of SHG is a good indicator of skin collagen content changed by radiofrequency treatment.[22] Thus, the 2D SHG image and total values that represent relative collagen concentration from the integration of all the pixel signals were obtained by MATLAB processing.

2.4 | Statistical analysis

2.4.1 | Phantom study

Every phantom was randomly measured on the surface 20 times by the DRS system, and collagen and coffee concentrations were derived from the diffuse reflectance. The results of the concentration from five skin-mimicking phantoms were divided into two groups. One

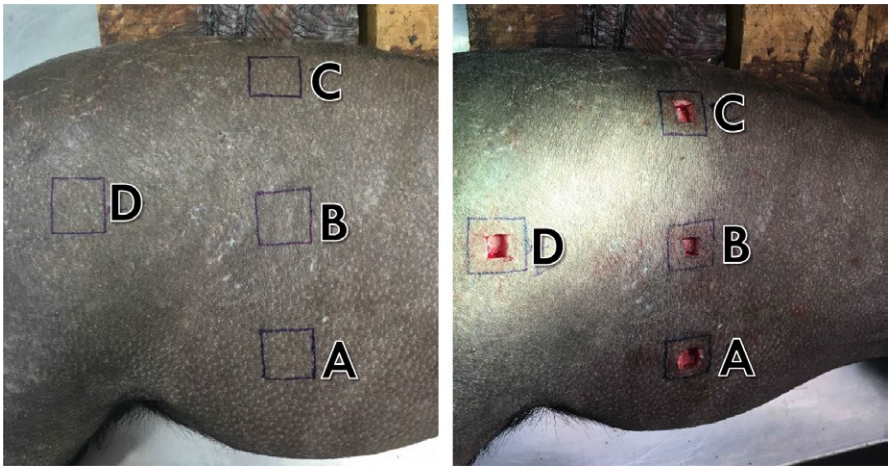

FIGURE 2 Photographs of experimental Lanyu pig before measurement and after cutting the skin tissue. Each black square is 3×3 cm^2. (A, abdominal skin; B, ventral skin; C, dorsal skin; D, pelvic skin) [Colour figure can be viewed at wileyonlinelibrary.com]

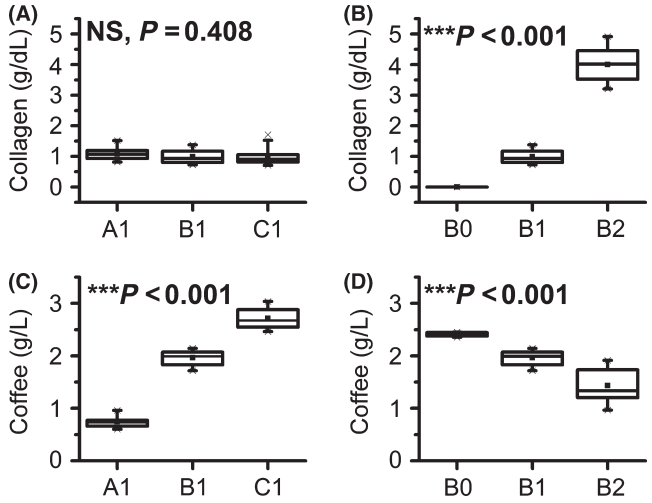

FIGURE 3 Boxplots of evaluation of (A, B) collagen and (C, D) coffee concentrations of gelatin phantoms by DRS system. The results of ANOVA analysis of coffee and collagen concentrations in different groups are shown in the figures. ***P<.001. NS, not significant. The data were presented as median line, mean (■), 25%-75% within boxes, and whole data range

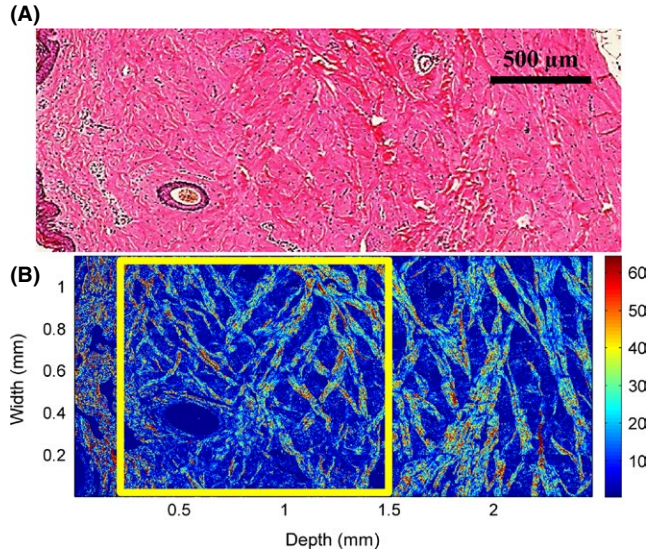

FIGURE 4 A, Representative Lanyu pig skin sections stained with hematoxylin and eosin and B, SHG image by post-processing with MATLAB [Colour figure can be viewed at wileyonlinelibrary.com]

group (A1, B1, and C1) had equivalent collagen concentrations but different coffee concentrations. On the contrary, the other group (B0, B1, and B2) had different collagen concentrations but an equivalent coffee concentration. The mean value and standard deviation (SD) were calculated (mean±SD). One-way analysis of variance (ANOVA) was used for the comparisons of each group.

2.4.2 | Animal study

Every site of the Lanyu pig skin was randomly measured 15 times in the black square by the DRS system, and collagen concentration was derived from the diffuse reflectance. To perform sound statistical analysis, we took eight SHG image sections from each skin site, and for each image, the intensity of all pixels were summed up to obtain a value to represent the collagen concentration. Correlation analysis was performed on two sets of values that represent collagen concentration through the DRS and SHG technologies. One-way ANOVA was also used in two groups.

OriginPro 2015 data analysis and graphing software (OriginLab Corporation, Northampton, MA, USA) was used in the statistical analysis and graphing for both studies.

3 | RESULTS

3.1 | Phantom study

The collagen and coffee concentrations of two phantom groups determined by the DRS system are shown in the boxplots in Figure 3. Other chromophore concentrations, such as gelatin, are not shown because no statistical difference between the groups is observed, and both groups have values close to the theoretical values. In groups A1, B1, and C1, the collagen concentrations are 1.08±0.18, 0.98±0.19, and 1.00±0.29 g/dL, respectively (Figure 3A), which are close to the theoretical value (1.0 g/dL) and have no significant difference (P=.408). However, the coffee concentrations in groups A1, B1, and C1 are 0.75±0.11, 1.96±0.13, and 2.71±0.19 g/L, respectively (Figure 3C), which are lower than the theoretical values. The interquartile ranges (IQRs) of coffee concentration increase when the coffee contents in the phantoms increase. The collagen concentrations in groups B0, B1, and B2 are 0±0, 0.98±0.19, and 4.0±0.55 g/dL, respectively (Figure 3B). B0 and B1 have concentrations close to theoretical values, but the concentration in B2 is lower than the theoretical values (5.0 g/dL). Nevertheless, only the coffee concentrations in the B0 phantom are close to the theoretical value of 2.5 g/L, and B1 and B2 phantoms are 1.96±0.19 and 1.43±0.29 g/L, respectively, which are lower than the theoretical values (Figure 3D). Figure 3B and D show that the IQR of collagen and coffee concentration also increase.

3.2 | Animal study

The representative histology and SHG image of the same Lanyu pig section are shown in Figure 4. The color bar represents the SHG intensity from the collagen in Figure 4B. Both figures display the epidermis, papillary region, and reticular region arranged from left to right. Nevertheless, compared to the H&E histology, the SHG intensity image from the collagen structure is clearer. To match the interrogation depth of detection by DRS system, we referred to our previous study, in which Monte Carlo simulation was used to determine the average penetration depth of photon packets with common optical properties.[23] According to different μ_a and μ_s' combinations, the average interrogation depth is 400-1400 μm. For the SHG intensity integration, we select 6×8 pieces of SHG image (Light Square in Figure 4B) whose depth is from 200 to 1523.44 μm. The boxplots (Figure 5) show the values that represent collagen concentrations of Lanyu pig at four skin sites by DRS and SHG. Positive correlation (r=.902) is observed

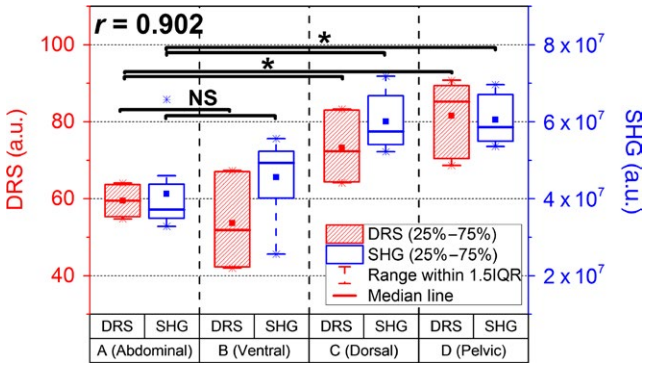

FIGURE 5 Comparison of collagen content of Lanyu pig by second-harmonics generation microscopy and diffuse reflectance spectroscopy system. The data were presented as median line, mean (■), 25%-75% within boxes, and whole data range. The correlation of two technologies is .902. *P<.05. NS, not significant [Colour figure can be viewed at wileyonlinelibrary.com]

in the collagen content detected by the two technologies, and both P<.001 by one-way ANOVA analysis. The lowest average collagen concentration occurs at the abdominal skin (A) and at the ventral skin (B) when measured with the SHG system and the DRS system, respectively. We found no statistic difference between A and B sites when using either measurement system. On the other hand, we observed significant differences between A and C sites and between A and D sites for both systems. In addition, the pelvic skin (D) has the highest average collagen content in either SHG or DRS measurements.

4 | DISCUSSION

In phantom study, the DRS system was sensitive and effective for the quantification of collagen concentration. No collagen value at B0 phantom was detected, and only slight influence of different coffee concentrations on groups A1, B1, and C1 was observed. A strong evidence from the DRS system illustrated that melanin has no effect on collagen concentration quantification. However, the measurements in the two phantom groups underestimated coffee concentration when collagen was present in the phantoms. In groups B0, B1, and B2, increasing collagen in the phantoms decreased coffee contents. On the contrary, the collagen concentration of B2 phantom was slightly underestimated at increased IQR. We speculated that the collagen pellets can affect the solubility of coffee in the phantom, and the phantoms were not mixed well. The collagen pellets were irregularly scattered on these phantom surfaces, because the collagen powder was not able to dissolve uniformly. Therefore, the variations of collagen and coffee concentration were getting larger due to increasing collagen concentration.

In animal study, we used SHG as a standard for the evaluation of collagen contents, and the evaluation results were compared with the in vivo skin collagen contents derived from the DRS system. These two well-known techniques are different in tissue probing scale and mechanism. To have a reasonable comparison between the results obtained from the two techniques, all SHG images were trimmed to cover depths from 200 to 1523.44 μm beneath the stratum corneum, which coincided with the average probing depth variation range of our DRS system. We observed significant differences between collagen contents of A and C sites and between A and D sites for both measurement systems. These findings agreed with the fact that it is commonly expected that the collagen concentration at dorsal (C) and hip (D) skin would be higher than that at the abdominal (A) skin. However, there is no significant difference between A and B sites in either measurement. This fact suggests that these two anatomical sites could have similar skin characteristics. In addition, the B site has a very wide IQR in DRS measurement and we speculate that the ventral skin could have relatively large structural variation. In fact, we found from skin biopsies that the dermis at C and A sites had largest and smallest thicknesses among all sites investigated, respectively, and the B site located between A and C sites had dermis thickness which varied between the largest and smallest dermis thickness values found at C and A sites. Since the probing region of the DRS technique contains tissue volumes located deeper than the average probing depth mentioned in the previous section, the high dermis thickness variation at the ventral skin could translate to the wide IQRs of DRS measurement results at that site.

In our previous study, we demonstrated that the DRS system can not only quantify collagen concentration but can also observe that the melanin may not interfere in collagen concentration recovery.[7,8,13] Here, we performed the gelatin phantom study to demonstrate that the coffee chromophore (similar to melanin) has no direct influence on collagen concentration recovery. Moreover, the Lanyu pig study shows high positive correlations between the DRS system and collagen evaluation of biopsy section with SHG microscopy evaluation. Besides, when compared with SHG microscopy collagen measurement in this study, the DRS system can not only detect collagen content at a depth of 400-1400 μm noninvasively but can also be convenient and time saving.

5 | CONCLUSION

Our DRS system provides a convenient, relatively low system cost, and time-saving tool that can be used for the evaluation of skin collagen content. In this study, the skin-mimicking phantoms and animal study demonstrated that collagen content can be accurately quantified and cannot be influenced by melanin-like chromophore. In addition, the evaluation of pig skin collagen content has high positive correlations with SHG microcopy. Our result demonstrated that our system has potential in clinical skin collagen evaluation. In the near future, we will employ our DRS system to assess the influence of aging or various skin treatments on skin collagen content variation.

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REFERENCES

1. Naylor EC, Watson RE, Sherratt MJ. Molecular aspects of skin ageing. *Maturitas*. 2011;69:249-256.
2. Moloney SJ, Edmonds SH, Giddens LD, Learn DB. The hairless mouse model of photoaging: evaluation of the relationship between dermal elastin, collagen, skin thickness and wrinkles. *Photochem Photobiol*. 1992;56:505-511.
3. Fligiel SE, Varani J, Datta SC, Kang S, Fisher GJ, Voorhees JJ. Collagen degradation in aged/photodamaged skin *in vivo* and after exposure to matrix metalloproteinase-1 *in vitro*. *J Invest Dermatol*. 2003;120:842-848.
4. Martin P. Wound healing–aiming for perfect skin regeneration. *Science*. 1997;276:75-81.
5. Lee JY, Yang CC, Chao SC, Wong TW. Histopathological differential diagnosis of keloid and hypertrophic scar. *Am J Dermatopathol*. 2004;26:379-384.
6. Tseng SH, Hsu CK, Lee JYY, Tzeng SY, Chen WR, Liaw YK. Noninvasive evaluation of collagen and hemoglobin contents and scattering property of *in vivo* keloid scars and normal skin using diffuse reflectance spectroscopy: pilot study. *J Biomed Opt*. 2012;17:077005.
7. De Vito JR, Merogi AJ, Vo T, Boh EE, Fung HK, et al. Role of *Borrelia burgdorferi* in the pathogenesis of morphea/scleroderma and lichen sclerosus et atrophicus: a PCR study of thirty-five cases. *J Cutan Pathol*. 1996;23:350-358.
8. Kikuchi K, Masuda Y, Yamashita T, Sato K, Katagiri C, et al. A new quantitative evaluation method for age-related changes of individual pigmented spots in facial skin. *Skin Res Technol*. 2016;22:318-324.
9. Koehler MJ, König K, Elsner P, Bückle R, Kaatz M. *In vivo* assessment of human skin aging by multiphoton laser scanning tomography. *Opt Lett*. 2006;31:2879-2881.
10. Liao YH, Kuo WC, Chou SY, Tsai CS, Lin GL, et al. Quantitative analysis of intrinsic skin aging in dermal papillae by *in vivo* harmonic generation microscopy. *Biomed Opt Express*. 2014;5:3266-3279.
11. Hsu CK, Tzeng SY, Yang CC, Lee JY, Huang LL, et al. Non-invasive evaluation of therapeutic response in keloid scar using diffuse reflectance spectroscopy. *Biomed Opt Express*. 2015;6:390-404.
12. Chen SY, Chen SU, Wu HY, Lee WJ, Liao YH, Sun CK. *In vivo* virtual biopsy of human skin by using noninvasive higher harmonic generation microscopy. *IEEE J Sel Top Quant*. 2010;16:478-492.
13. Ha RY, Nojima K, Adams WP Jr, Brown SA. Analysis of facial skin thickness: defining the relative thickness index. *Plast Reconstr Surg*. 2005;115:1769-1773.
14. Tseng SH, Bargo P, Durkin A, Kollias N. Chromophore concentrations, absorption and scattering properties of human skin *in vivo*. *Opt Express*. 2009;17:14599-14617.
15. Chen YW, Tseng SH. Efficient construction of robust artificial neural networks for accurate determination of superficial sample optical properties. *Biomed Opt Express*. 2015;6:747-760.
16. De Grand AM, Lomnes SJ, Lee DS, Pietrzykowski M, Ohnishi S, et al. Tissue-like phantoms for near-infrared fluorescence imaging system assessment and the training of surgeons. *J Biomed Opt*. 2006;11:014007.
17. Bauer EA, Uitto J. Collagen in cutaneous diseases. *Int J Dermatol*. 1979;18:251-270.
18. Nishidate I, Aizu Y, Mishina H. Estimation of melanin and hemoglobin in skin tissue using multiple regression analysis aided by Monte Carlo simulation. *J Biomed Opt*. 2004;9:700-710.
19. Sullivan TP, Eaglstein WH, Davis SC, Mertz P. The pig as a model for human wound healing. *Wound Repair Regen*. 2001;9:66-76.
20. Kuo TY, Lin MJ, Chen LR, Huang LLH. Lanyu Minipig is a better model system for studying wound healing. *J Biomater Tiss Eng*. 2015;5:886-894.
21. Lin CY, Lien CH, Cho KC, Chang CY, Chang NS, et al. Investigation of two-photon excited fluorescence increment via crosslinked bovine serum albumin. *Opt Express*. 2012;20:13669-13676.
22. Tsai TH, Lin SJ, Lee WR, Wang CC, Hsu CT, et al. Visualizing radiofrequency-skin interaction using multiphoton microscopy *in vivo*. *J Dermatol Sci*. 2012;65:95-101.
23. Chen YW, Guo JY, Tzeng SY, Chou TC, Lin MJ, et al. Toward reliable retrieval of functional information of papillary dermis using spatially resolved diffuse reflectance spectroscopy. *Biomed Opt Express*. 2016;7:542-558.

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