Journal of Biomedical Optics

BiomedicalOptics.SPIEDigitalLibrary.org

Reflectance spectroscopy for noninvasive evaluation of hair follicle stage

Caihua Liu
Yue Guan
Jianru Wang
Xiewei Zhong
Xiuli Liu
Dan Zhu

SPIE.

Reflectance spectroscopy for noninvasive evaluation of hair follicle stage

Caihua Liu,[a,b] Yue Guan,[a,b] Jianru Wang,[a,b] Xiewei Zhong,[a,b] Xiuli Liu,[a,b,*] and Dan Zhu[a,b,*]
[a]Huazhong University of Science and Technology, Wuhan National Laboratory for Optoelectronics, Britton Chance Center for Biomedical Photonics, 1037# Luoyu Road, Wuhan 430074, China
[b]Huazhong University of Science and Technology, Department of Biomedical Engineering, MoE Key Laboratory for Biomedical Photonics, 1037# Luoyu Road, Wuhan 430074, China

Abstract. Hair follicle offers an excellent model for systems biology and regenerative medicine. So far, the stages of hair follicle growth have been evaluated by histological examination. In this work, a noninvasive spectroscopy was proposed by measuring the diffuse reflectance of mouse skin and analyzing the melanin value. Results show that the skin diffuse reflectance was relatively high when hair follicles were at the telogen stage and at the beginning of the anagen stage, and decreased with the progression of the anagen stage. When the hair follicle entered into the catagen stage, the diffuse reflectance gradually increased. The changes in the melanin content of skin had contrary dynamics. Substages of the hair follicle cycle could be distinguished by comparing the changes in melanin value with the histological examination. This study provided a new method for noninvasive evaluation of the hair follicle stage, and should be valuable for basic and therapeutic investigations on hair regeneration. [DOI: 10.1117/1.JBO.20.5.051011]

Keywords: hair follicle cycle; reflectance spectroscopy; melanin value; histological examination.

Paper 140407SSPRR received Jun. 25, 2014; accepted for publication Oct. 30, 2014; published online Nov. 27, 2014.

1 Introduction

Hair follicles possess multiple independent cell populations including stem cells, dermal papilla cells, and epithelial cells, with distinct locations and functions.[1] The cycling feature of telogen (hair rest), anagen (hair growth), and catagen (hair involution) throughout postnatal life for morphogenesis remodeling makes the hair follicle an excellent model for systems biology, regenerative and translational medicine.[2–5] The discrimination of the hair follicle stage is mostly based on morphological characters revealed by histological and immunohistochemical techniques, but the samples need to be removed from the body. Naked-eyes are used to observe the transformation of skin color with hair follicle cycling, but it is empirical and equivocal.[6] Additionally, the natural hair cycle domain and regenerative wave are different for different mice due to individual differences, and even desynchronized in different regions of an individual animal,[7] which makes it difficult to precisely evaluate the hair follicle stage in animal model establishment and therapeutic assessment. Hence, it is particularly urgent to develop a reliable method to noninvasively distinguish the hair follicle stage.

The optical techniques show some prospects to noninvasively detect the structure information of hair follicles.[8–10] For example, the colorimetry has been used to measure *de novo* hair regeneration based on three terms, including light intensity ($L*$), green to red ($a*$), and blue to yellow ($b*$) color bands, but it was also invasive because the skin samples should be cut off from the experimental animals.[8] Confocal microscopy has also been used to image the entire intact hair follicle, but it could only focus on one *ex vivo* hair follicle, with a very limited view.[9] Two-photon microscopy has recently been used to monitor cell behaviors during physiological hair regeneration in live mice, yet with only revelation of the cellular mechanisms at the very beginning of the anagen stage.[10]

Actually, diffuse reflectance spectroscopy has been successfully applied to investigate *in vivo* skin.[11–16] The reflectance spectrum carries information of the tissue structure and components, for instance, the chromospheres' concentration such as melanin and hemoglobin.[11–14] Besides, reflectance spectroscopy documents information of topical skin area, which should afford a promising tool for distinguishing interindividual variation.[15]

In this study, a commercial optical fiber spectrometer was used to measure the diffuse reflectance of mouse skin during a hair follicle cycle, then the melanin value of skin was calculated based on the spectroscopy. In addition, the macroscopic appearance of the skin with hair follicle cycling was observed and a histological examination was performed. Finally, the comparison of the histological examination with the measurements was applied to evaluate the stage of the hair follicle.

2 Materials and Methods

2.1 *Animal Model and Experimental Protocol*

This work was approved by Huazhong University of Science and Technology Institutional Review Board for Animal Study. Female C57BL/6 mice ($n = 30$), 3 to 3.5 weeks old, with the first postnatal telogen hair follicles,[6] were obtained from Experimental Animal Center of Wuhan University (Wuhan, China) and fed under standard rodent feeding conditions. After 3 days of environmental adaptation, animals were intraperitoneally anesthetized with a mixture of chloral hydrate (0.02 g/ml) mixed with ethylurethanm (0.1 g/ml). To avoid possible effects of depilatory cream on the hair growth or cycle, the eudipleural areas of dorsal hair were shaved off by

*Address all correspondence to: Xiuli Liu, E-mail: xlliu@mail.hust.edu.cn; Dan Zhu, E-mail: dawnzh@mail.hust.edu.cn

an electric shaver and then cleared carefully with a razor blade. In the next 23 days, the mice were photographed and the reflectance spectra of the back skin were measured every day.

2.2 *Histopathological Examination*

To canonically discriminate the stage of hair follicle growth, histological examination was performed. Skin biopsies of mice dorsal skin were cut at different durations (3 to 7 weeks old). Three mice were sacrificed at each time point and the dorsal skin samples were detached and then fixed with 4% neutral formaldehyde and dehydrated with graded alcohol. After paraffin embedding, the samples were sliced with a thickness of 4 to 5 μm and then stained with hematoxylin and eosin (HE). The slice was imaged by a microscopy (Nikon Ni-E, Nikon, Japan) equipped with a color digital industrial camera (DS-Fi2, Nikon, Japan).

2.3 *Visible-Near-Infrared Fiber Spectrometer*

To noninvasively obtain the change in mice skin with hair cycling, a visible-near-infrared (VIS-NIR) fiber spectrometer (USB-4000, Ocean Optics, Florida) was used to measure the diffuse reflectance spectra of *in vivo* mouse skin.[12] The system is composed of a light source (HL-2000), a reflection probe (R400-7), and a spectrometer as well as computer software. Before the experiment, the system was standardized with a reflectance standard film made up of barium sulfate. During this experiment, the probe was kept vertical and soft touched to the *in vivo* dorsal skin, then the data were saved. For each mouse, the eudipleural areas of dorsal skin were measured, respectively; for each area, the reflectance spectrum was measured three times and then averaged. Further quantification of reflectance was analyzed at a wavelength of 480 nm because of the most obvious and disciplinary changes in reflectance as well as the relatively low absorption of whole blood.[17]

2.4 *Pigmentation Evaluation of Mice Skin*

Hemoglobin and melanin are major pigmentations in the skin. During hair follicle growth cycling, the skin color is changed from pink to gray, and then to black, which reflects different melanin contents in the skin. However, the absorption of hemoglobin will decrease sharply in the wavelength range of dark red or NIR, which is much less than that of melanin. In this work, the melanin value (dimensionless) was calculated from reflectance at 660 and 880 nm as follows:[18,19]

$$M_{\mathrm{v}} = \frac{500}{\log\ 5} \times \left(\log\ \frac{R_{880}}{R_{660}} + \log\ 5 \right). \tag{1}$$

Here, R_{660} and R_{880} represent the reflectance at the red (660 nm) and the NIR (880 nm) regions, respectively.

2.5 *Quantitative Analysis*

In order to quantitatively investigate the changes in reflectance and pigmentation of mouse dorsal skin with hair follicle cycling, the relative changes in reflectance (ΔR_{rel}) at 480 nm and the melanin value ($\Delta M_{\mathrm{v\text{-}rel}}$) were obtained based on Eqs. (2) and (3), respectively. Here, R_0 and M_{v0} are the initial values on the first day of the experiment (D1), and R_{t} and M_{vt} are the values at different days during a hair follicle cycle.

$$\Delta R_{\mathrm{rel}} = \frac{R_{\mathrm{t}} - R_0}{R_0}, \tag{2}$$

$$\Delta M_{\mathrm{v\text{-}rel}} = \frac{M_{\mathrm{vt}} - M_{\mathrm{v0}}}{M_{\mathrm{v0}}}. \tag{3}$$

3 Results

3.1 *Macroscopical Changes in Mouse Dorsal Skin With Hair Follicle Cycling*

Figure 1 show typical macroscopical changes in mouse dorsal skin during a hair follicle cycle. The dorsal skin is pink on the first day of the experiment (D1), which is at the beginning of the anagen stage. In the following several days, the color of the skin gradually changes from pink-to-gray. On the fifth day, the dorsal skin of the mouse seems to be gray. The skin color continues to darken as the anagen stage progresses, i.e., the dorsal skin shows dusty blue from the seventh to eighth day and dark blue from 11th to 15th day. Afterward, the skin color brightens to light blue (D18), gray-to-pink (D21), and finally back to pink (D23). Such cycling changes last for 23 days and roughly reflect the three stages of hair follicle growth, including anagen, catagen, and telogen. It can also be found that the skin color shows a synchronous change for both the tested area and the surrounding eudipleural areas.

3.2 *Morphological Changes During a Hair Follicle Cycle*

Figure 2 shows the skin tissue histological examination with HE staining. Since the morphological feature can precisely classify

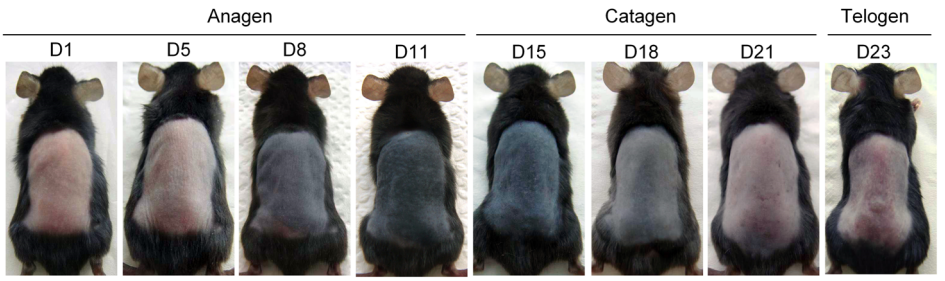

Fig. 1 Macroscopical changes in mouse dorsal skin with hair follicle cycling. D means Day, D1 means the first day of experiment, which also corresponds to the starting date after hair follicles has transmitted to anagen stage, Dn means the day of the experiment. The speculated stages of hair follicle are generally shown as anagen, catagen, and telogen.

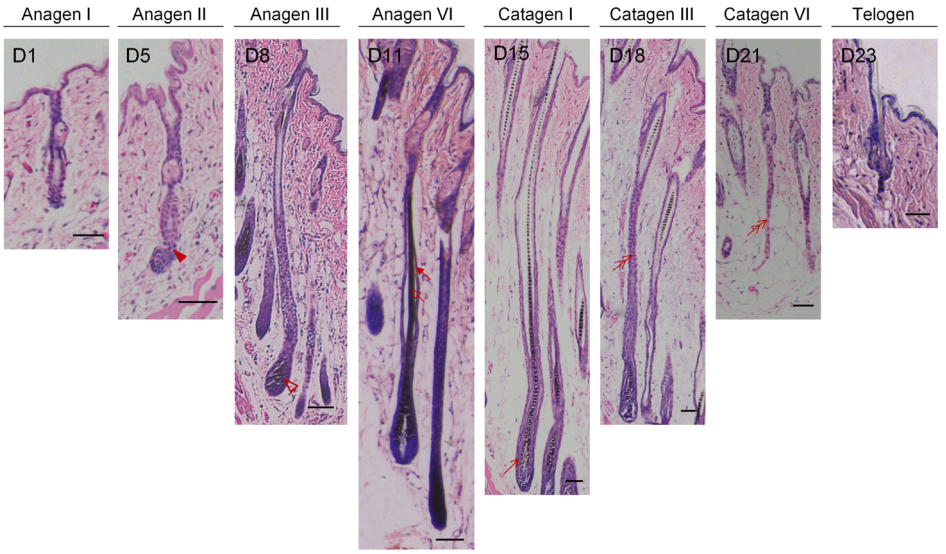

Fig. 2 Development of the morphology of a hair follicle at different days and stages with hair follicle cycling. The solid arrow head indicates proliferating keratinocytes; the hollow arrow head indicates an inflated hair bulb; the solid and hollow triangular arrows indicate ORS and IRS, respectively; the hollow arrow reveals apoptosis of the hair matrix; the double hollow arrow reveals apoptosis of the cycling part of the hair follicle. Results were examined by HE staining. Scale bar: 100 μm.

the stage of the hair follicle, the substages of the hair follicle cycle are indicated. On the first day of the experiment, the hair follicle is very short and is only located in the dermis. The faint expansion of dermal fibroblasts suggests that the hair follicle is at the anagen I. On the fifth day, the keratinocyte strands between the dermal papilla and club hair thicken with an obvious hair germ (Fig. 2, solid arrow head), which means that the hair follicle enters into the anagen II. On the eighth day, the hair follicles extend to the subcutis with an inflated hair bulb (Fig. 2, hollow arrow head). On the 11th day, the hair follicles reach to the bottom of the thickened adipose tissue with distinct dermal papilla, an outer root sheath (ORS) (Fig. 2, solid triangular arrow) and an inner root sheath (IRS) (Fig. 2, hollow triangular arrow), which means that hair follicles are from the anagen III to anagen VI. From the 15th to 18th day, the hair follicles reside in the deep subcutis, but the hair bulb gradually shrinks due to apoptosis of the matrix cells (Fig. 2, hollow arrow), which reveals the onset of the catagen stage. Afterward, further cell apoptosis leads to the absence of a multilayered structure (Fig. 2, double hollow arrows), and then enters into the telogen stage of the hair follicle.

3.3 *Typical Changes in Reflectance Spectrum of Mouse Skin During a Hair Follicle Cycle*

Typical reflectance spectra of mouse skin were measured within a hair follicle cycle [Fig. 3(a)]. On the first day, the reflectance of skin is very high; it begins to decrease sharply in 5 days. The reflectance spectra are at a low level from the 11th to 13th days. From the 15th day of the experiment, the reflectance begins to increase and reaches the initial level on the 23rd day. For higher reflectance spectra, obvious absorption peaks in the range of 500 to 600 nm can be found; whereas the absorption peaks almost disappear when the reflectances are at a lower level.

Further, relative changes in skin reflectance (480 nm) on different days compared with the first day were calculated. Since three mice were sacrificed for histological examination after every batch of measurements, only nine mice were left on the 23rd day after seven batches of measurement. Figure 3(b) shows the mean value and deviation from 9 to 30 mice. The results show that the relative change in reflectance is not obvious for the first few days; and then a decrease of 13% to 83% is shown from the 5th to 11th day. In the next a few days, the reflectance remains in a constant state. From the 15th day on, the reflectance begins to increase sharply and on the 23rd day is almost same as that on the first day.

3.4 *Skin Melanin Contents at Substages of Hair Follicle Cycle*

Based on the reflectance spectra, the melanin values in skin on different days of the experiment were extracted using Eq. (1). Figure 4(a) shows that the melanin contents have a relatively low level during the first five days of the experiment, then increase rapidly and reach a relatively high level on the 11th day. Over the next several days, the melanin values reduce sharply and return to the initial value on the 23rd day.

Further, the changes in melanin content relative to the first day of the experiment were calculated. After being compared with the results from the histological examination, the relative changes in melanin content at substages of the hair follicle cycle are indicated in Fig. 4(b). The melanin content in skin has very little change at the anage-I of the hair follicle cycle. On the fifth day, there is a 6% increase in the melanin value, which means that the hair follicle growth enters into the anagen II. Then the hair follicle growth quickly changes from the anagen III to IV on the seventh to ninth days. On the 11th day, the hair follicles enter into a later stage of growth (anagen VI) and the melanin value has an increase of >60%. Once the hair follicles enter into the catagen stage (on the 15th to 21st days), the melanin value decreases rapidly, and goes down to the minimum value when hair follicles fall into the telogen stage (on the 23rd day).

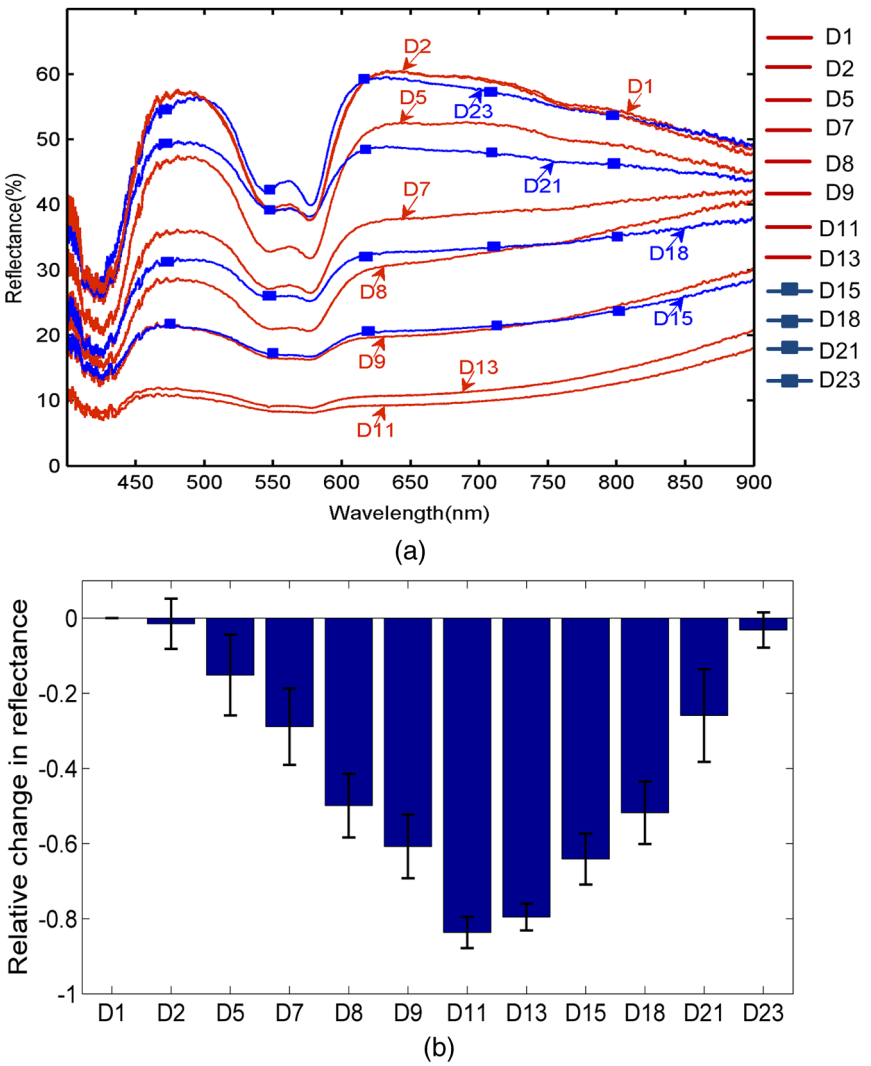

Fig. 3 Typical diffuse reflectance spectra (a) and its relative change ($\lambda = 480$ nm) (b) of mouse skin with hair follicle cycling. Text arrows indicate different days (abbreviated as D) during a hair follicle cycle. Solid lines and lines with square indicate the decreased and increased trend of reflectance spectrum from the 1st to 13th and 15th to 23rd day, respectively. Data are shown as mean ± SD ($n \geq 9$).

4 Discussion

Generally, the hair follicle of mouse skin is roughly divided into three stages, i.e., anagen, categen, and telogen.[1,6] It is well known that the skin color of a mouse changes with the hair follicle cycle, but the change is sometimes too small to be discriminated by naked eyes. Histological examination with HE staining has been a gold standard to determine the hair follicle cycle, including the classification of the substages of the hair follicle.[6]

In this study, reflectance spectroscopy was proposed to noninvasively obtain the dynamical pigmentation of skin during a hair follicle cycle. Compared with the histological examination, the reflectance spectra can also be used to quantify the hair follicle stage within a cycle by an *in vivo* measurement method. Compared with the direct observation with naked eyes, the measurements of the reflectance spectra could provide a more sensitive change. For instance, the skin color shows no obvious difference between the first day and fifth day, but the spectra are easy to distinguish; this may indicate the onset of anagen. In addition, a similar color can be observed at the catagen or the anagen stage, and the different stages of a hair follice can be easily distinguished by continuous measurement *in vivo*. Thus, the reflectance spectroscopy is a promising tool with which to distinguish the states of hair follicle cycle.

Compared to the reflectance spectrum, the melanin content in skin is very closely related to the stage of hair follicle growth. It is well know that the reflectance spectrum of skin carries information about the skin composition, such as melanin and hemoglobin.[11–15] In this work, the melanin contents in skin were extracted by reflectance spectroscopy. The melanin content increases with the progress of anagen, and decreases with the transition of catagen and telogen. This is due to the fact that the melanocytes in the hair bulb produce more melanin to pigment the hair shaft and the surrounding skin once the hair follicles enter into the anagen stage, while they promote cell apoptosis once the hair follicle regresses and rests.[20] Therefore, melanin is a significant indicator of the hair follicle cycle. In this study, the melanin content in skin was calculated according to the reflectance at 660 and 880 nm which was determined with the principle of the widely used skin evaluator Mexameter® MX18 (Courage + Khazaka electronic GmbH, Köln, Germany).[18,19] It is well known that whole blood has a

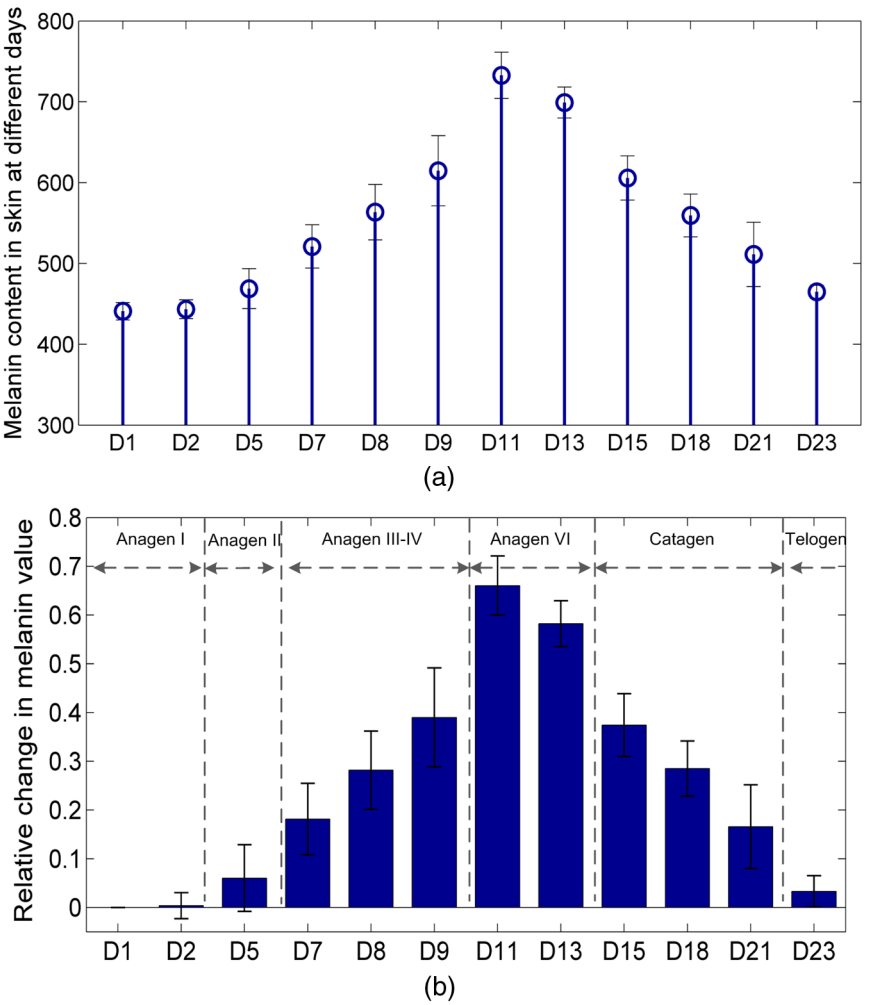

Fig. 4 The skin melanin value calculated from reflectance spectroscopy during a hair follicle cycle (a). Relative change in melanin content on different days and corresponding substages of the hair follicle cycle (b). Data are shown as mean ± SD ($n \geq 9$). Stages of hair follicles are subdivided into anagen I, II, III–IV, and VI, catagen, and telogen.

weak absorption in the wavelength range of dark red or NIR light, so the measured reflectance at 660 and 880 nm could indicate the melanin content in skin. Actually, other wavelengths, such as 650 and 710 nm could also be effective for the evaluation of melanin.[21] The melanin value during a complete hair follicle change is in the range of 440 to 730, which is consistent with the instructional melanin range of brown skin, dark hair (150 to 500) and black skin (600 to 999) in the MX® 18 instructions. The melanin in the anagen skin is 1.7 times that of telogen skin, which also agrees with the melanin content of 1.7 to 3.2 times in black hair compared with light brown hair.[22]

Even though reflectance spectroscopy has shown a potential for the evaluation of hair growth, further investigations need to be performed to determine whether the method itself has an effect on the hair follicle cycle. It is worth noting that the absorption peaks of spectra change with the hair follicles' cycling, and even disappear when the reflectance spectra are at a relatively low level. This is because the wavelength range corresponds to the absorption peaks of blood. At later anagen and early catagen stages, light can hardly penetrate into the dermis because of the strong absorption of the concentrated melanin pigment, so it is impossible to detect the information from blood vessels located in dermis. In contrast, when hair follicles are at the later catagen, telogen, and early anagen stages, the melanin content in the skin epidermis and hair shaft is much lower. More light can enter into the dermis and be absorbed by blood, so the characteristic peak of blood can be clearly demonstrated in the reflectance spectra. In addition, blood perfusion may be related to the hair follicle cycling. Angiogenesis happened during the synchronized switches of the hair follicle from telogen to anagen, and the degeneration of capillary loops was noted during the catagen stage.[23,24] However, with the increase in absorption of melanin, reflectance spectroscopy is insufficient to describe the relationship of blood perfusion and the cycle of hair follicle, therefore, further investigation should be performed in the future.

5 Conclusion

In this work, a commercial NIR-VIS fiber spectrometer was used to measure skin reflectance at different stages of a hair follicle cycle. The reflectance of skin was relatively high when the hair follicle stayed at the telogen stage, and began to decrease once the hair follices entered the anagen stage. Then it increased again when the hair follices moved to the catagen stage. The changes of melanin content and those of reflectance spectrum are contrarieties. Compared the change in the melanin value

with the histological examination, the substages of a hair follicle cycle could be distinguished. This study provided a new method for noninvasive evaluation of the hair follicle stage and should be valuable for long-term tracing and assessment of hair-related treatment.

Acknowledgments

This study was supported by the National Nature Science Foundation of China (Grant Nos. 81171376, 91232710, and 812111313), the Science Fund for Creative Research Group (Grant No. 61121004), and the Research Fund for the Doctoral Program of Higher Education of China (Grant No. 20110142110073).

References

1. R. Paus and G. Cotsarelis, "The biology of hair follicles," *N. Engl. J. Med.* **341**(7), 491–497 (1999).
2. M. V. Plikus et al., "Self-organizing and stochastic behaviors during the regeneration of hair stem cells," *Science* **332**(6029), 586–589 (2011).
3. Y. Al-Nuaimi et al., "The cycling hair follicle as an ideal systems biology research model," *Exp. Dermatol.* **19**(8), 707–713 (2010).
4. C. Mason and P. A. Dunnill, "A brief definition of regenerative medicine," *Regen. Med.* **3**(1), 1–5 (2008).
5. C. M. Chuong et al., "Physiological regeneration of skin appendages and implications for regenerative medicine," *Physiology* **27**(2), 61–72 (2012).
6. S. Müller-Röver et al., "A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages," *J. Invest. Dermat.* **117**(1), 3–15 (2001).
7. M. V. Plikus et al., "Analyses of regenerative wave patterns in adult hair follicle populations reveal macro-environmental regulation of stem cell activity," *Int. J. Dev. Biol.* **53**(5–6), 857–868 (2009).
8. T. Y. Tzung et al., "Colorimetry provides a rapid objective measurement of de novo hair growth rate in mice," *Skin Res. Technol.* **15**(4), 459–463 (2009).
9. J. E. Kloepper et al., "Point scanning confocal microscopy facilitates 3D human hair follicle imaging in tissue sections," *Exp. Dermatol.* **19**(7), 691–694 (2010).
10. P. Rompolas et al., "Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration," *Nature* **487**(7408), 496–499 (2012).
11. L. E. Dolotov and Y. P. Sinichkin, "Features of applying fiber-optic sensors in spectral measurements of biological tissues," *Opt. Spectrosc.* **115**(2), 187–192 (2013).
12. E. Drakaki et al., "Spectroscopic methods for the photodiagnosis of nonmelanoma skin cancer," *J. Biomed. Opt.* **18**(6), 061221 (2013).
13. A. E. Karsten et al., "Diffuse reflectance spectroscopy as a tool to measure the absorption coefficient in skin: South African skin phototypes," *J. Photochem. Photobiol.* **89**(1), 227–233 (2013).
14. X. Zhong, X. Wen, and D. Zhu, "Lookup-table-based inverse model for human skin reflectance spectroscopy: two-layered Monte Carlo simulations and experiments," *Opt. Express* **22**(2), 1852–1864 (2014).
15. C. H. Liu et al., "Enhancement of skin optical clearing efficacy using photo-irradiation," *Laser Surg. Med.* **42**(2), 132–140 (2010).
16. F. Ries et al., "Estimation of skin phototypes with optical parameters: an experimental study using newly developed fibre optic detection device," *Int. J. Cosmet. Sci.* **35**(1), 50–56 (2013).
17. V. V. Tuchin, *Handbook of Optical Biomedical Diagnostics*, SPIE Press, Bellingham, Washington (2002).
18. H. Kato et al., "A prospective randomized controlled study of oral tranexamic acid for preventing postinflammatory hyperpigmentation after Q-switched ruby laser," *Dermatol. Surg.* **37**(5), 605–610 (2011).
19. K. Yoshimura et al., "Usefulness of a narrow-band reflectance spectrophotometer in evaluating effects of depigmenting treatment," *Aesthetic Plast. Surg.* **25**(2), 129–133 (2001).
20. E. K. Nishimura, "Melanocyte stem cells: a melanocyte reservoir in hair follicles for hair and skin pigmentation," *Pigm. Cell Melanoma Res.* **24**(3), 401–410 (2011).
21. L. E. Dolotov et al., "Design and evaluation of a novel portable erythema-melanin-meter," *Laser Sur. Med.* **34**, 127–135 (2004).
22. A. N. Bashkatov et al., "Estimate of the melanin content in human hairs by the inverse Monte-Carlo method using a system for digital image analysis," *Quantum Electron.* **36**(12), 1111–1118 (2006).
23. L. Mecklenburg et al., "Active hair growth (anagen) is associated with angiogenesis," *J. Invest. Dermatol.* **114**(5), 909–916 (2000).
24. K. Yano, L. F. Brown, and M. Detmar, "Control of hair growth and follicle size by VEGF-mediated angiogenesis," *J. Clin. Invest.* **107**(4), 409–417 (2001).

Caihua Liu received her BS degree in biotechnology from Huazhong University of Science and Technology (HUST) in 2008, and PhD degree in biomedicine engineering from HUST in 2014. Now, she is a research fellow at the Britton Chance Center for Biomedical Photonics, HUST. Her current research interests include tissue optical clearing, laser-tissue interaction, tissue repair and regeneration.

Dan Zhu is currently a full professor and the deputy director of the Division of Biomedical Photonics, Wuhan National Optoelectronics Laboratory. She has authored more than 100 papers in the field of tissue optics, optical diagnosis, and therapy principle and techniques. In recent years, she has been focusing on optical clearing of tissue *in vivo* for improving optical imaging.

Biographies of the other authors are not available.