Monitoring Photoaging by Use of Multiphoton Fluorescence and Second Harmonic Generation Microscopy

Sung-Jan Lin[1,2], Shiou-Hwa Jee[1], Jung-Yi Chan[3], Ruei-Jr Wu[4], Wen Lo[4], Hsin-Yuan Tan[2,5], Wei-Chou Lin[6], Jau-Shiuh Chen[1], Tai-Horng Young[2], Chih-Jung Hsu[1]*, Chen-Yuan Dong[3]*

1. Department of Dermatology, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan.
2. Institute of Biomedical Engineering, National Taiwan University, Taipei, Taiwan
3. Department of Dermatology, Cathay General Hospital, Taipei, Taiwan
4. Department of Physics, National Taiwan University, Taipei, Taiwan.
5. Department of Ophthalmology, Chang Gung Memorial Hospital, Linko, Taiwan
6. Department of Pathology, National Taiwan University, Taipei, Taiwan

Abstract

It is a field of great interest to develop therapies to rejuvenate photoaged skin. However, the treatment response can not be ideally determined due to lack of a reliable non-invasive method to quantify photoaging. In this study, the photoaging process of skin is investigated by use of a multiphoton fluorescence and second harmonic generation microscopy. We obtain the autofluorescence and second harmonic generation images of superficial dermis from facial skin of individuals of different ages. The results show that autofluorescence signals increase with age while second harmonic generation signals decrease with age. The results are consistent with the histological findings in which collagen is progressively replaced by elastic fibers. In the case of severe photoaging, solar elastosis can be clearly demonstrated by the presence of thick curvy autofluorescent materials in the superficial dermis. We propose a second harmonic generation to autofluorescence aging index of dermis to quantify the photoaging changes. This index is shown to be a good indicator of photoaging. Our results suggest that multiphoton fluorescence and second harmonic generation microscopy can be developed into a non-invasive imaging modelity for the clinical evaluation of photoaging.

Keywords: multiphoton, second harmonic generation, photoaging, collagen, elastic fiber, autofluorescence

*Address correspondence to: CJH cz038@ms29.hinet.net and CYD cydong@phys.ntu.edu.tw

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1. Introduction

Skin aging can be divided into two types: intrinsic aging and photoaging. Intrinsic aging is universal and correlated with ages. There is decrement of cellular functions associated with intrinsic aging. On the other hand, intrinsic aging can be superimposed by photoaging which is caused by the cumulative sun light exposure.[1] The ultraviolet spectrum of sun light is believed to play a major role in causing the cutaneous photoaging. Dryness, irregular pigmentation, and wrinkling are the clinical features of skin photoaging. In addition to pigmentary changes of epidermis, photoaging can be evaluated by examination of changes of extracellular matrix component in the superficial dermis histologically.[1] Due to various cytokines and increased enzyme activities, collagen content in the dermis decreases with photoaging and elastic fiber content increases. In severe cases of photoaging, increased disorganized elastic materials can be demonstrated in the superficial dermis (solar elastosis). A number of surgical procedures and topical medication are used to rejuvenate photoaged skin aimed at remodeling collagen and elastic fibers in the superficial dermis, including laser resurfacing,[2] intense pulse light treatment,[3] chemical peeling,[4] and topical tretinoids.[1] For the determination of treatment effect, repeated biopsies and protein extraction and electrophoresis are required to quantified the changes in extracellular matrix proteins. In addition to the tedious work involving protein extraction, evaluation of the treatment response can only be evaluated via subjective clinical assessment by the physicians and patients, because there is lack of a reliable non-invasive method to quantify the dermal photoaging.

Over the past few years, mutliphoton fluorescence (MPF) microscopy has become popular in the biomedical imaging.[5,6] The non-linear excitation of fluorescence photons using ultrafast, near- infrared excitation sources have important advantages in its ability to acquire enhanced axial depth discrimination images, reduced overall specimen photodamage, and increased imaging penetration depths. In addition to morphological information, characteristic autofluorescence (AF) from various cells and luminescence from components of the extracellular matrix can help to specifically feature subjects of interest.[7] Compared with collagen fibers, elastic fibers are more effective in generating multiphoton autofluorescence when a longer excitation wavelength is used.[8]

In addition to multiphoton autofluorescence, a non-vanishing second order susceptibility can contribute to a second harmonic generation (SHG) signal in

biological structures lacking an inversion symmetry. Collagen, muscle fibers and microtubules have been shown to be effective in generating second harmonic signals.[7,9-11] Collagen is a particularly interesting subject for SHG imaging due to the wide distribution of collagen in extracellular matrix of various tissues including skin. Due to the special molecular structures of collagen, we have shown that SHG imaging can be used to monitor the heat-induced structural transitions of collagen.[12]

In this work, we combine MPA and SHG images to study photoaging. In comparison with histological results, our results show that MPA can be used to quantify the elastic fiber while SHG can be used to quantify the collagen components of skin. We propose a second harmonic generation to autofluorescence aging index of dermis to quantify the photoaging of skin.

2. Materials and methods

The multiphoton fluorescence and SHG microscopic system used in this study is a modified version of a home-built laser scanning microscopic imaging system based on an upright microscope (E800, Nikon, Japan) described previously.[13] A diode-pumped (Millennia X, Spectra Physics, Mountain View, CA), titanium-sapphire (ti-sa, Tsunami, Spectra Physics) was used as the excitation source. The 760 nm output of the ti-sa laser is scanned in the focal plane by a galvanometer-driver x-y mirror scanning system (Model 6220, Cambridge Technology, Cambridge, MA). Prior to entering the upright microscope, the laser is beam expanded to ensure overfilling of the objective's back aperture. For high resolution imaging, a high numerical aperture, oil immersion objective (S Fluor 40x, NA 1.3, Nikon) was used. To direct the expanded laser spot to the sample, a short-pass dirochoic mirror (700DCSPRUV, Chroma Technology, Brattleboro, VT) is used to reflect the incident excitation laser source. The average laser power at the sample is 5.5 mW. The generated autofluorescence and SHG signals are collected by the same objective, and pass through the dichroic onto the photo-detectors. Prior to reaching the detectors, the autofluorescence and SHG signals are separated by a secondary dichroic mirror (435DCSX, Chroma Technology). The SHG signal centered at 380nm was reflected by the secondary dichroic and further filtered using a bandpass filter (HQ380/20, Chroma Technology) while the longer wavelength autofluorescence passes through the dichroic mirror and a broad-band pass filter (E435LP**,** Chroma Technology) before being detected. The signal photons were processed by a single-photon counting PMT (R7400P, Hamamatsu, Japan) and a home-built discriminator.

The facial skin specimens were obtained from the cheeks of three patients undergoing facial cosmetic surgery aged 20, 40 and 70 years respectively. A thin slice of formalin-fixed samples is mounted on the slide and covered with a No. 1.5 coverslip for the MP and SHG imaging. A large area scan along the dermal-epidermal junction composed of a 3 by 3 array of neighboring images was serially acquired and assembled. Five 100μm by 200μm rectangular areas just below the epidermis are randomly chosen in each case. In each rectangular area, the pixels of second harmonic generation are defined as **A** and pixels of autofluorescence are defined as **B**. The second harmonic generation to autofluorescence aging index of dermis (**SAAID**) is defined as **(A-B)/(A+B)**. The index of each rectangular area is computed and the average index in each case is calculated. For comparison, the specimens are further processed for histological examination using hematoxylin and eosin stains and elastic stains (Verhoeff-van Gieson stain). In hematoxylin and eosin stains, elastin and collagen have varying degrees of pink staining and severe solar elastosis is characterized by bluish gray curvy fibers in the superficial dermis. In elastic stains, elstic fibers are stained blue-black and collagen fibers are red.

3. Results and discussion

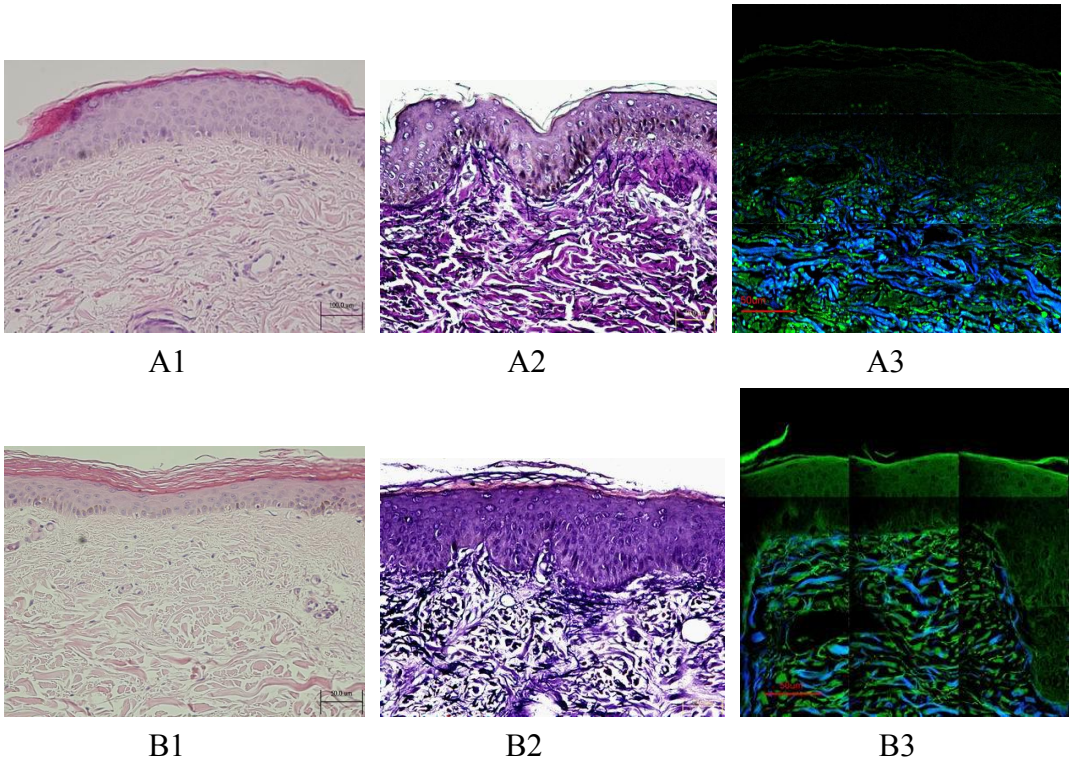
The H&E stains, elastic stains and MPA & SHG images of the three cases are shown in figure 1. With aging, areas of autofluorescence signals increase while SHG signals decrease in the superficial dermis. In the patient of 20 years old, SHG signals and AF signals are evenly mixed in the dermis. In the patient of 40 years old, proportions of AF signals increase in the dermis and SHG signals decrease. In the patient of 70 years old, the dermis is almost composed of autofluorescence and scanty SHG signals can only be revealed in the papillary dermis.

A similar trend of the changes of proportion of elastic tissue and collagen fibers is demonstrated in the histological findings. In H&E stains, elastic tissue can not be clearly differentiated in patients of 20 and 40 years old. The elastic stains show slight increase of elastic tissue in the patient of 40 years old. In the patient of 70 years of age, solar elastosis characterized by the presence of curvy bluish fibers is observed in H&E staining (arrow). This observation is further confirmed by the elastic stains in which a large amount of black elastic tissue is present in the superficial dermis (arrow). The areas of elastic tissue in the histology are consistent within the areas of autofluorescence in the MPA&SHG images. Hence, in our setting, MPA can be used to represent the elastic tissue while SHG signals reflect the presence of collagen in the

dermis.

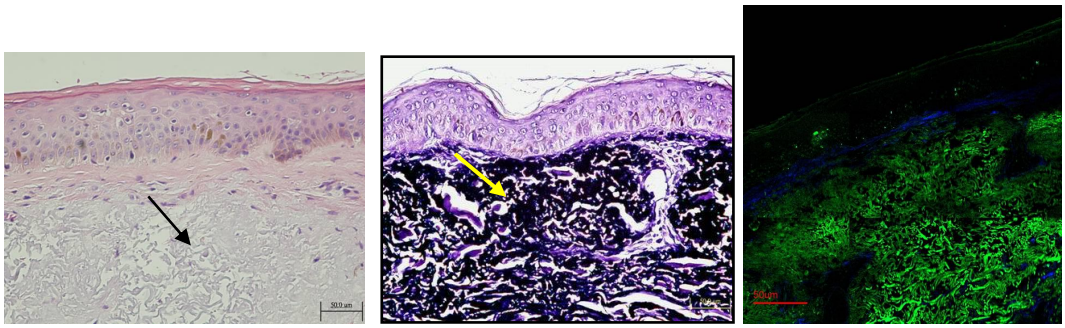
To better quantify the changes of elastic and collagen fibers, the SAAID is shown in figure 2. Consistent with the above mentioned findings, SAAID decreases with age, showing the trend of increasing elastic component of dermis with photoaging. In the case of the 70 years old, the average SAAID is -0.93, indicating the collagen fibers are almost completely replaced by elastic fibers.

In conclusion, multiphoton fluorescence and second harmonic generation microscopy can be used to evaluate the cutaneous photoaging process. The elastic fibers are effective in generating autofluorescence signals and SHG signals reflect the presence of collagen fibers in the dermis. In the severe case of photoaging, solar elastosis can be clearly demonstrated by the presence of masses of autofluorescent materials in the superficial dermis. An index of photoaging SAAID can be used to quantify the degrees of photoaging. Our results suggest that multiphoton fluorescence and second harmonic generation microscopy can be further developed into a non-invasive imaging tools for the clinical evaluation of photoaging.



A1 A2 A3

B1 B2 B3



C1 C2 C3

Figure 1. The hematoxylin and eosin stains (A1, B1, C1), elastic stains (A2, B2, C2) and multiphoton and second harmonic generation images (A3, B3, C3; second harmonic signals are blue and autofluorescence signals are green) of the three cases (20 years old: A1-A3; 40 years old: B1-B3; 70 years old: C1-C3). Bars: 50μm.

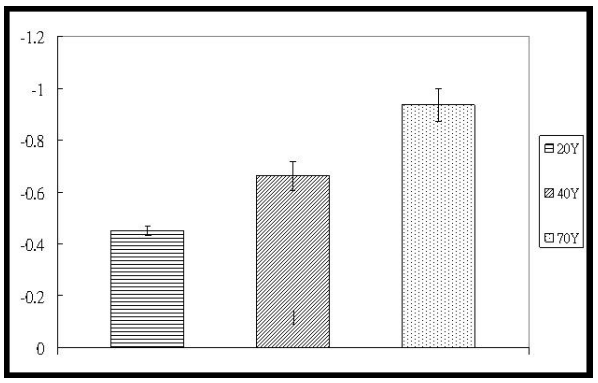


Figure 2. SAAID of the three cases.

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Address correspondence to Dr. Chih-Jung Hsu cz038@ms29.hinet.net and Dr. Chen-Yuan Dong at cydong@phys.ntu.edu.tw.

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