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Autofluorescence Loss in Photobleaching for Human Dentin *ex vivo*

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Two-photon fluorescence microscopy was performed on the enamel-dentin junction area of a human tooth using a femtosecond pulsed laser. We obtained a clear image contrast between the bright dentin and dark tubules with the autofluorescence generated from the endogenous fluorophores in dentin. The autofluorescence shows a broad spectrum due to complex cross links between dentinal collagens, which extend from blue to orange wavelengths (470–590 nm), but a gradual autofluorescence loss in photobleaching was observed for a long-term exposure under strong excitation. For increasing excitation power, we found that two-step decay becomes significant in the spectrally integrated autofluorescence.

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I. INTRODUCTION

Currently, fluorescence microscopy is widely used for biological imaging with various high-resolution techniques. Since most biological specimens require an extra process of dying for fluorescence, the toxicity of fluorescent dye molecules becomes an issue. A few different techniques are applied to avoid toxicity, such as second harmonic generation (SHG) imaging. However, the SHG imaging requires specimens with a crystalline and well-aligned structure to acquire images with good quality. Securing the structural homogeneity of a biological specimen often requires an extra chemical process. Due to this limitation, autofluorescence has gained much attention as an alternative method. Collagen is known to show autofluorescence and is contained in human teeth. Although the entire mechanism of dentinal autofluorescence has not been clarified yet, the cross link of collagens is considered the main origin [1–4]. Since the dentin of human teeth contains collagen-I, autofluorescence microscopy can be used for dentinal studies [5–9].

However, it is known that autofluorescence images are vulnerable to laser excitation. The fluorescence was suppressed for long-term exposure to laser excitation, resulting in poor image contrast, and even permanent damage was

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often observed with intense excitation. After the absorption of pulsed laser excitation, the initially excited carriers relaxed into the ground state. During the intra-relaxation, the excess energy of the excited carriers gives rise to various non-radiative processes, during which molecular vibrations are enhanced, and the carriers are trapped by various non-radiative states. When a biological specimen is exposed to strong laser excitation for a long time, the intra-relaxation processes likely modify the photochemical environment such as the molecular structure and fluorescence energy levels. Consequently, photo-bleaching occurs with a decreased fluorescence efficiency [10–13], and the phenomenon is studied by fluorescence loss with the photobleaching (FLIP) method. Nevertheless, the photo-bleaching in dentinal collagens has rarely been investigated.

FLIP is a highly efficient tool to evaluate the photo-bleaching of specimens, where repetitive scanning of the same region of interest (ROI) gives rise to a gradual decrease in average fluorescence intensity [14–17]. In this work, we obtained auto-fluorescence from a human tooth (molar) and a two-photon microscopy image of the enamel-dentin junction. We also found that the auto-fluorescence is gradually suppressed through FLIP. The fluorescence decay tended to behave as a two-step decay process with increasing excitation power. Therefore, we were able to obtain both fast and slow decay times against initial excitation power. These results will provide important conditions for fluorescence microscopy on human teeth.

II. METHODS

A human tooth (molar) was donated by Pusan National University Dental Hospital for the purpose of scientific research. Since the optical experiment on the extracted tooth was harmless and noninvasive regarding the donor, this research was exempt for pre-experiment IRB approval; PNUDH-2020-036 Pusan National University Dental Hospital. It was kept in saline solution at 5 ℃, then dried at room temperature for 72 hours and stored in a resin solution for 24 hours. Finally, the tooth sample became embedded in the solidified transparent resin, as shown in Fig. 1(a). The enamel outside inhibits the resin medium from percolating inside. A slice of the tooth was obtained using a diamond cutter as shown in Fig. 1(b), and the sample surface was polished with sandpaper. To remove possible residues, we cleaned the slice with distilled water and dried it again for 72 hours at room temperature. Figure 1(c) shows an image of the prepared sample, where the enamel area is distinguishable from the dentin area.

In Fig. 1(d), the fluorescence via two-photon absorption is shown schematically. Although the one-photon energy of the fundamental laser wavelength is transparent to the

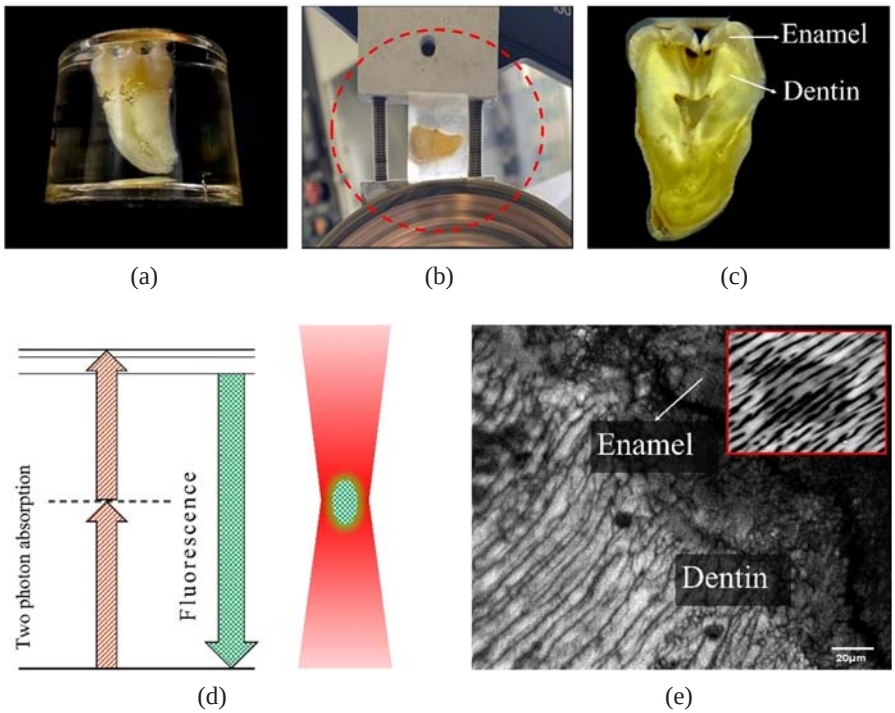

FIG. 1. Human tooth sample preparation and two-photon fluorescence microscopy: (a) a human tooth (molar) is stored in clear resin, (b) a layer is obtained through a longitudinal cutting process, (c) confocal microscope image of a cross-sectional tooth area attached to a slide glass, (d) fluorescence (dotted) via two-photon absorption (hatched) is generated only at the intense area of a focused laser spot, by which an improved spatial resolution is achieved, and (e) two-photon fluorescence microscope image of the dentin-enamel junction area, where auto-fluorescence is generated from the collagen in dentin (bright), while the dentinal tubules are seen dark. Inset shows a partial suppression of auto-fluorescence in the dentin (photo-bleached).

bandgap, absorption can be induced with a large excitation intensity, where the transition energy of the nonlinear optics corresponds to twice the one-photon energy. Two-photon fluorescence occurs only in the relatively intense area of a spot, while one-photon fluorescence appears over the whole excited spot. Therefore, the spatial resolution of two-photon fluorescence microscopy is significantly improved by avoiding unwanted excitation.

To induce a two-photon absorption in the dentin area, a Ti:sapphire laser with a central wavelength of 780 nm was used, which generates 200 fs pulses at an 80 MHz repetition rate. The resolution of an image is inversely proportional to the excitation wavelength. To find the optimal wavelength condition, we tested excitation wavelengths of 680 nm to 1064 nm. We concluded that an excitation wavelength of 780 nm shows optimal brightness and resolution. For repetitive fast scanning, the scanning time and ROI were controlled by a Galvano mirror system and objective lens. Slowing the scanning speed leads to an increase in the dwell time of each pixel. A higher dwell time offers higher brightness, but also it causes photodamage to the specimen. Therefore, we acquired 0.55 seconds for each image with a dwell time of 0.03 ms. The fast scanning speed gives higher temporal resolution in the FLIP measurement. The poor quality due to low brightness was compensated by collecting multiple images of the same ROI. The fluorescence signal was separated by bandpass filters into three different channels and detected by photomultiplier tubes (PMTs). In Fig. 1(e), a two-photon fluorescence microscope image was obtained from the dentin-enamel junction area, where the endogenous collagen in dentin generates auto-fluorescence. Since the dentinal tubules are hollow, they appear as dark lines. In the magnified inset image of Fig. 1(e), a dark square area is seen where the fluorescence is suppressed compared to the surroundings. This can be attributed to a photobleaching section. The selected ROI was investigated by the FLIP experiment.

We also measured the auto-fluorescence spectrum of dentin under a direct one-photon excitation of 404 nm, which was generated by a picosecond pulsed laser operating at a repetition rate of 40 MHz. The excitation power (~150 μW) was moderate, and the focused beam spot diameter was 1 μm.

III. RESULTS & AND DISCUSSION

As shown schematically in Fig. 2(a), photobleaching occurs gradually when fluorophores are exposed to strong excitation. Some fluorophores lose their original nature, and the number of active fluorophores decreases. In Fig. 2(b), the FLIP in two-photon fluorescence microscope images was observed under 74 mW excitation for the 780 nm central wavelength. To have a single image of the same ROI, 0.55 seconds was necessary for scanning. For the initial few seconds of laser excitation, most of the collagen fluorophores were activated. The bright dentin (red) and the

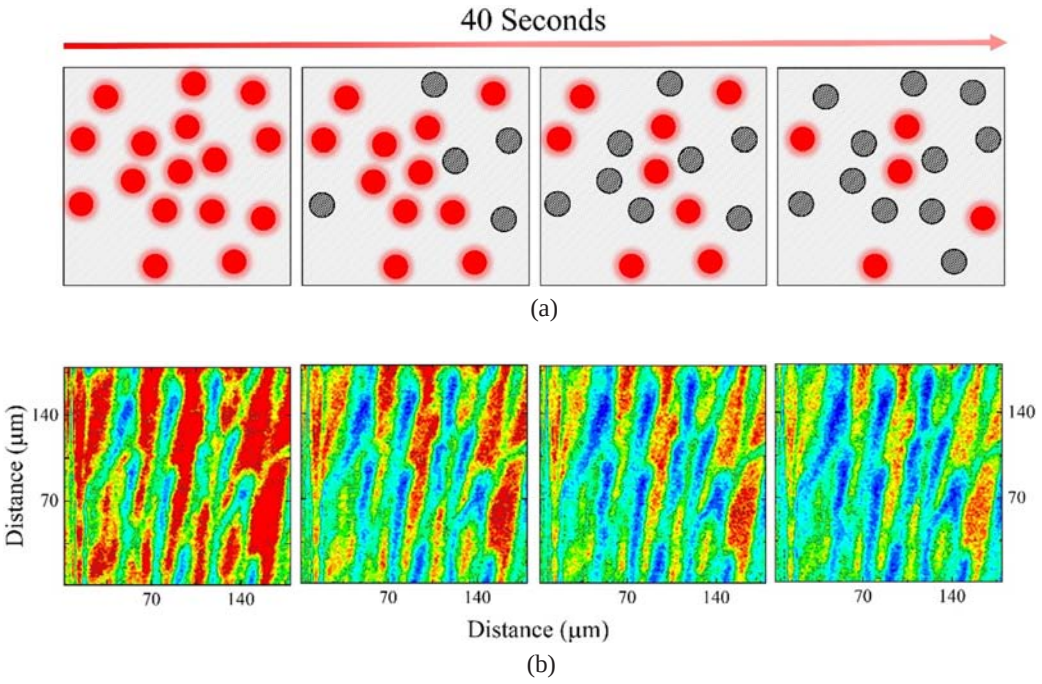

FIG. 2. Fluorescence lost in photobleaching and two-photon fluorescence microscope images of human dentin: (a) as a consequence of long-term exposure to strong excitation, photobleaching occurs gradually, where some fluorophores lose their original feature (dashed) and the number of active fluorophores decreases and (b) the fluorescence loss with the photobleaching (FLIP) measurement with a two-photon fluorescence microscope. The images were obtained for 40 seconds under 0.55 seconds of each scanning. The dark tubules and bright dentin are shown in blue and red, respectively.

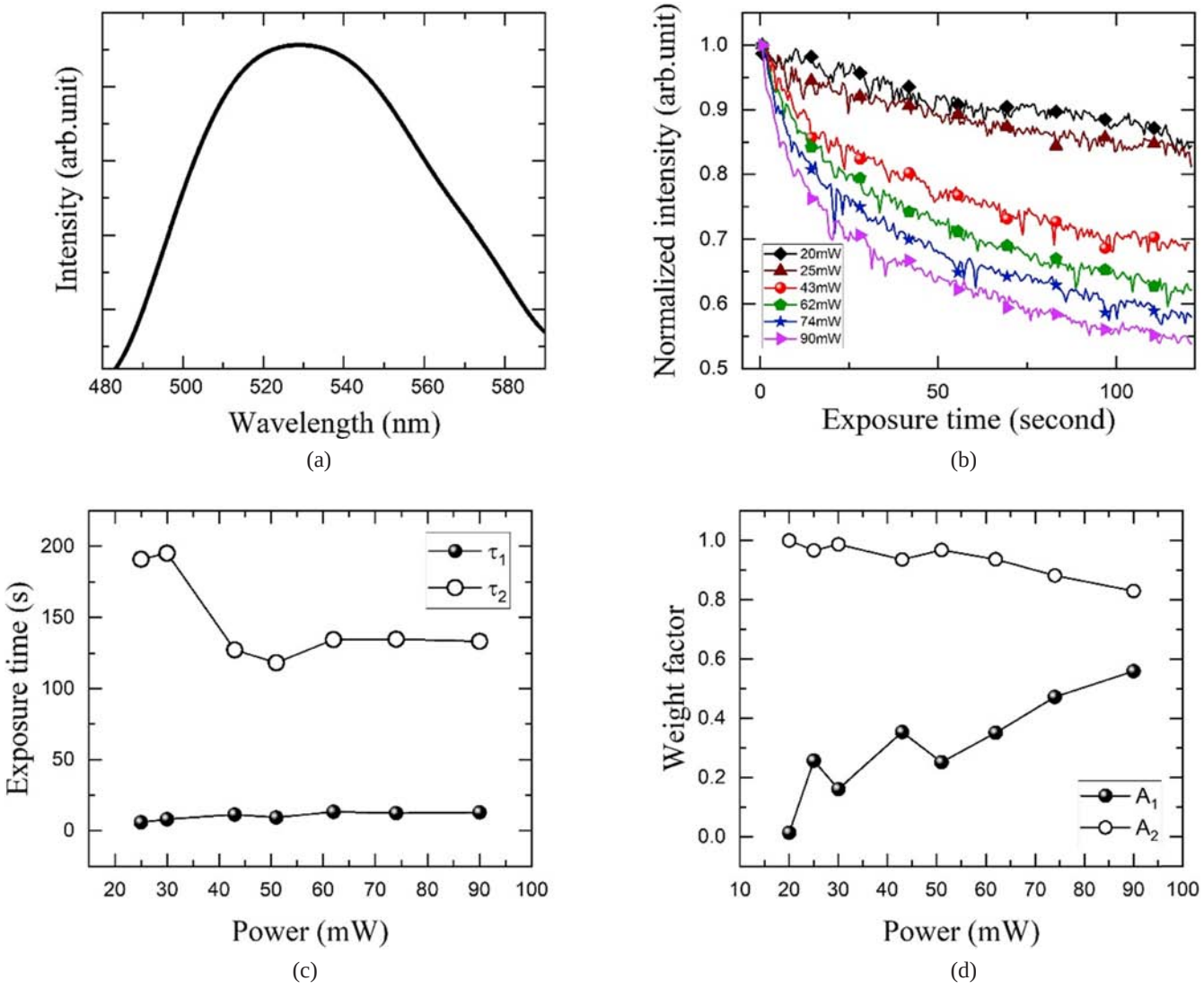

FIG. 3. Excitation power dependence fluorescence loss with the photobleaching (FLIP) measurement in human dentin: (a) autofluorescence spectrum of human dentin, (b) for increasing excitation power, the photobleaching occurs significantly, where a gradual suppression of fluorescence was observed up to 200 seconds, (c) excitation power dependence of the two characteristic times, where τ_1 and τ_2 are fast and slow decay times, respectively, and (d) as excitation power increases, the weight factor of the fast component (A_1) is enhanced, but the weight factor of the slow component (A_2) decreases.

hollow tubules (blue) show a clear contrast in the image. However, as the scanning was repeated for 40 seconds, we observed that the image contrast gradually deteriorated. It was suggested that the cross link of dentinal collagen and chemical compositions such as tyrosine, tryptophan [2, 3], and pyridinoline [4] could emit fluorescence, but the exact autofluorescence mechanism is still unknown due to the complexity of endogenous fluorophores in dentin.

In Fig. 3(a), we found that the dentinal collagen gives rise to a broad fluorescence spectrum due to the complex endogenous fluorophores of the dentinal collagen, which extends from blue (~480 nm) to yellow (~580 nm) via the dominant green (~530 nm). When a two-photon fluorescence image was obtained, we used a PMT. Therefore, the image intensity is determined through spectral integration. To enhance the contrast of a microscope image, one may increase the detection integration time. In this case, a whole image takes a long measurement time for spatial scanning. The other may often increase excitation power instead of the detection integration time. However, for both increasing excitation power and exposure time, it likely that more fluorophores are photobleached.

In Fig. 3(b), we found that gradual fluorescence suppression occurs for increased excitation where we used the spectrally integrated autofluorescence. The normalized intensity decay proceeds up to 200 seconds. For the spectral integration over the broad autofluorescence spectrum, we used three detection channels of PMTs. The fluorescence signal was separated by bandpass filters. Each separated signal was detected by the three PMTs. which were opti-

mized near 460 nm, 520 nm, and 590 nm, respectively. We also considered the relative signal ratio of the three PMTs. The multiple endogenous fluorophores have different photobleaching damage thresholds. As excitation power increases, the one with the higher damage threshold will start to decay. Consequently, the result shows that the double exponential decay becomes significant at higher excitation power. For quantitative analysis, we used a fitting function:

$$I = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + I_0, \quad (1)$$

where the fast and slow decay terms were characterized with the two fitting parameters of decay time (τ_1 and τ_2) and weight factor (A_1 and A_1), respectively. We also used an offset (I_0) for the background signal. The excitation power dependence of the two decay times and weight factors are shown in Figs. 3(c) and 3(d). With 20 mW excitation power, the normalized intensity shows a monotonic decay. Therefore, there is no contribution of the fast decay term (A_1 = 0), and only the slow decay time (τ_2 − 180 s) was obtained. However, the fast component emerges with 25 mW excitation power. With increasing excitation power, A_2 shows a gradual increase. At the same time, the weight factor of the slow decay term (A_1) decreases. Interestingly, the fast decay time (τ_1 – 10 s) barely depends on excitation power. The slow decay time (τ_2) is shortened when excitation power is larger than 40 mW but remains near ~130 s when the excitation power is larger than 62 mW fluorophores to be photobleached.

The main advantages of autofluorescence imaging are its label-free and chemically nontoxic nature. However, the damage from interaction between the specimen and high flux of photons has hardly been studied. Therefore, in this study, we have measured the photon-induced damage of a two-photon FLIP process on human dentin. The decay of signal intensity over time suggests a potential hazard of the two-photon microscopy process. Moreover, the double exponential decay of human dentin over 25 mW of excitation implies the existence of two or more endogenous molecules with different populations that are being damaged during the process [18]. However, further study on photobleaching simulation and spectroscopy is required to determine the photobleaching thresholds of each individual fluorophore.

IV. CONCLUSION

We found that dentinal collagen generates a broad autofluorescence spectrum, which extends from blue (~480 nm) to yellow (~580 nm) via the dominant green (~530 nm). Therefore, two-photon autofluorescence microscopy can be a useful tool to investigate the structure of human teeth; the hollow tubules are seen as dark branches in the bright collagen-rich dentin with clear contrast without chemical toxication. However, we found that photobleaching occurs when the dentin is exposed to strong excitation through fluorescence loss in the photobleaching method. The intensity of fluorescence decreased exponentially over time with 0.03 ms of dwell time. The gradual suppression of autofluorescence proceeds up to 200 seconds, and the decay occurs in a double exponential manner at high excitation intensities. The results suggest that acquiring an autofluorescence signal under strong excitation not only decreases image quality over time but also damages multiple endogenous fluorophores.

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