

Imaging of laser-excited tissue autofluorescence bleaching rates

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Experimental methodology for imaging of laser-excited tissue autofluorescence bleaching rates has been developed and clinically tested. The fluorescence images were periodically captured from the same tissue area over a certain time, with subsequent detection of the fluorescence intensity decrease rate at each image pixel and further imaging the planar distribution of those values. Spectral features at each image pixel were analyzed with a hyperspectral imaging camera. Details of the equipment and image processing are described as well as some measurement results that confirm the feasibility of the proposed technology. © 2009 Optical Society of America

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1. Introduction

Fluorescence spectrometry is widely used to characterize living tissues noninvasively. Tissue autofluorescence (AF) can originate from numerous fluorescent proteins, flavins, porphyrins, and other chromophores with specific absorption and emission spectral bands. Laser-excited tissue AF spectra have been studied extensively over recent decades, especially the spectra related to skin [1–4]. Several authors have observed bleaching (fading) of the whole *in vivo* skin AF spectrum during laser irradiation; such studies dealt with spectra excited at UVA (N₂ laser, 337 nm), violet (diode laser, 405 nm), blue (Cd⁺ laser, 442 nm), green [diode-pumped solid-state (DPSS) laser, 532 nm], and red (He–Ne laser, 633 nm; diode laser, 670 nm) wavelengths, within the laser power range from 1 to 500 mW [5–8]. Double-exponential AF intensity decay over several minutes was recorded in most cases, indicating optical wash-out of fluorophores at the superficial layer(s) of skin. A practical aspect that generates interest in this phenomenon is the potential application for noninvasive

clinical diagnostics, since healthy and pathologic areas of skin show different values of the AF fading parameters [8].

The tissue AF photobleaching mechanism is still under discussion; however, one can assume that each tissue surface component (e.g., a chromophore cluster) might have its own specific AF bleaching rate, and any changes in the surface chromophore composition would induce corresponding changes in the AF bleaching rates. If so, analysis of the AF bleaching parameters could provide indirect information about the chromophore clusters and their distribution over the tissue surface. In the case of skin, such clusters are most probably located in the epidermal layer with a typical thickness of 0.1–0.2 mm.

With regard to specific tissue chromophores that are directly involved in the AF bleaching process, different unique combinations of chromophores might play a major role at different excitation wavelengths. Unfortunately, data available in the literature on absorption and emission bands of various skin chromophores [9–15] are contradictory. Therefore the processes leading to skin AF bleaching at any particular laser excitation wavelength, as well as the possibility to identify the involved chromophores, are still topics under investigation.

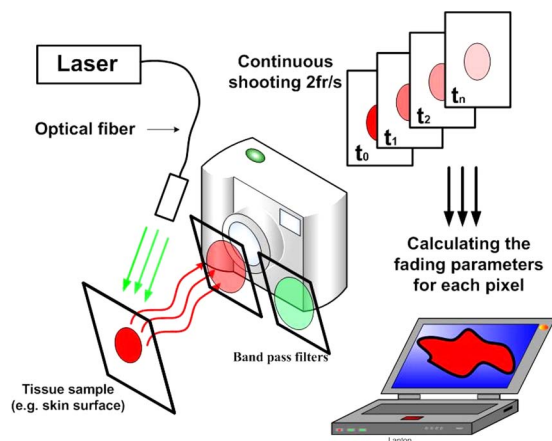


Fig. 1. (Color online) Setup for imaging the tissue AF bleaching (fading) rates.

Here we continue our previous research [8], with the aim to study more deeply the tissue (skin, nail) AF bleaching effects under continuous laser irradiation at two visible wavelengths (405 and 532 nm) by means of two imaging techniques. A technology for imaging the AF intensity bleaching rates using a standard CCD camera and self-made software has been developed. The hyperspectral imaging technique also has been approbated to obtain additional spectral information from separate pixels or pixel groups of such parametric images. The developed experimental methodology is described here along with some results of clinical measurements that confirm the feasibility of the proposed technique.

2. Method and Equipment

A. CCD Camera Setup

The setup for imaging the laser-induced AF bleaching rates with a CCD camera is presented in Fig. 1. It comprises two commercial cw lasers (supplied by B&W Tek, Newark, Delaware, USA), BWB-405-40-PIG-200-0.22-SMA, emitting the 405 nm line, and BWT-532-15-SMA, emitting the 532 nm line. The laser radiation was delivered to tissue by a 600 μm silica core optical fiber to ensure flexibility and increased uniform irradiation. Bandpass filters were used to select the maxima wavelengths of the AF spectra (550 nm in the case of 405 nm excitation and 630 nm in the case of 532 nm excitation, a digital CCD camera (Canon EOS 400D, 10.1 Mpixels, 3 Hz frame rate) and a laptop computer. The fluorescence images were captured twice per second over 20–30 s for determination of the τ_1 values, and over ~ 3 min for determination of the τ_2 values [see Eq. (1)], with subsequent analysis of fluorescence intensity decrease at each image pixel of the CCD camera. Laser power density on the tissue surface varied in the range from 1 to 10 mW/cm^2 , the area of the laser-excited surface was $\sim 4 \text{ cm}^2$. A Coherent laser check powermeter was used to control the fiber output power levels during irradiation. The CCD camera was positioned $\sim 20 \text{ cm}$ from the tissue surface

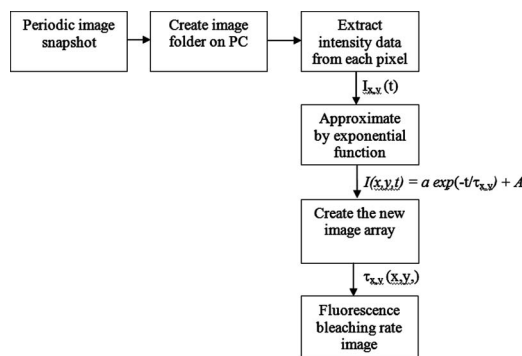


Fig. 2. Algorithm of the image processing for fast AF bleaching analysis.

during the measurements. As the irradiation was switched on, the camera began to collect a series of subsequent AF images. The set of images was further processed by use of an originally developed program; the processing algorithm is illustrated in Fig. 2.

In the case of fast bleaching analysis, the algorithm for image processing included the following steps:

1. Periodic capture of the set of spectrally filtered AF images over a fixed period of time.
2. Creation of an image library.
3. Extraction of intensity I_{xy} data from all the pixels and creation of the intensity-time-coordinate dependent array: $I_{xy}(t)$.
4. Fit the obtained temporal dependence of intensity bleaching at each pixel to the exponential function: $I(x,y,t) = a \exp(-t/\tau_{xy}) + A$, with subsequent determination of the corresponding τ_{xy} value for each image pixel. The fit was performed by least-squares analysis [16,17].
5. Collection of the bleaching time constants τ_{xy} from each single pixel and creation of a new parametric image array: $\tau_{xy}(x,y)$.
6. Creation of the image that represents planar distribution of the laser AF bleaching rates τ_{xy} by application of a specific color to each interval of the bleaching rate values.

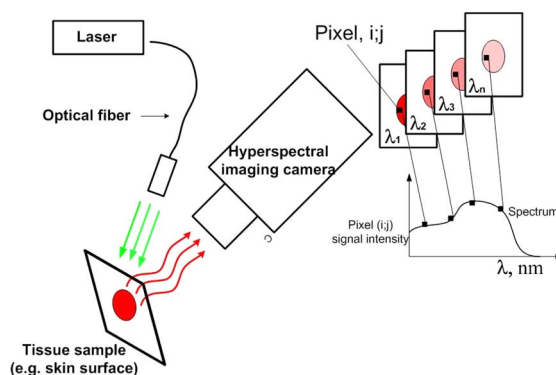


Fig. 3. (Color online) Scheme of the setup for hyperspectral imaging of the tissue AF bleaching.

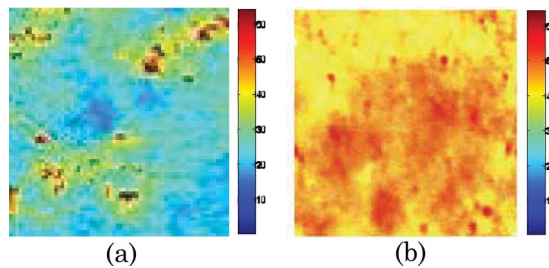


Fig. 4. (Color online) Distribution of the fast AF bleaching rate (τ_1) values across a $2 \times \text{cm} \times 2 \text{cm}$ healthy skin area: (a) 405 nm laser excitation and (b) 532 nm laser excitation. The color scale (at right) represents τ_1 values in seconds, in multiples of 10.

Surface distribution of the slow AF fading rates was obtained in a similar way. In this case step 4 comprised fitting the data (taken for a longer time, e.g., 2–3 min) to the double-exponential bleaching expression

$$I(x, y, t) = a \exp(-t/\tau_1) + b \exp(-t/\tau_2) + A, \quad (1)$$

with subsequent extraction of both fast and slow decay rates τ_1 and τ_2 . Again, the least-squares fitting technique was used. The processing algorithm was implemented in a MATLAB R2007a environment. Generally, a four-dimensional data set is created, comprising two dimensions of the pixel coordinates (x, y), AF intensity at each pixel, and time of each snapshot. Taking into account possible tissue movements during the measurements, all the images were stabilized with respect to the reference points on the measurement area; the estimated stabilization error was 7 ± 3 pixels.

B. Hyperspectral Imaging Setup

Spectral analysis of the tissue fluorescence registered at each image pixel was performed by means of a hyperspectral imaging camera (Fig. 3). We used the multispectral imaging system Nuance 2.4 (Cambridge Research and Instrumentation, Woburn, Massachusetts, USA) comprising an integrated CCD camera (1392×1040 pixels) and liquid crystal tunable filter (up to 500 bands in the 450–950 nm spectral region). The spectral resolution of the system was $\sim 2 \text{ nm}$ with a frame rate of 100 Hz.

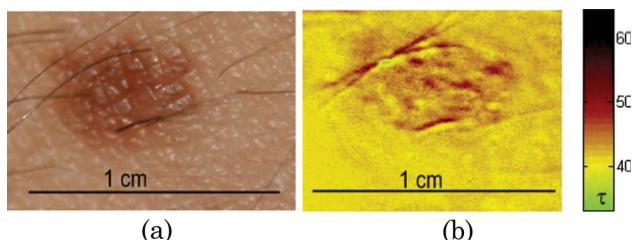


Fig. 5. (Color online) Medium-pigmented skin nevi: (a) ordinary photography and (b) the 532 nm laser fluorescence bleaching rate image. The color scale at right represents τ_1 values in seconds in multiples of 10.

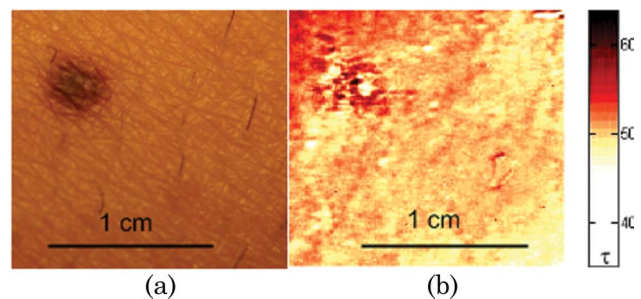


Fig. 6. (Color online) Hyperpigmented skin nevi: (a) ordinary photography and (b) the 532 nm laser fluorescence bleaching rate image. The color scale at right represents τ_1 values in seconds in multiples of 10.

3. Results of the Measurements

A. Healthy Skin: Single Spot Measurements and AF Bleaching Rate Images

As a continuation of the skin single spot ($\sim 4 \text{ mm}^2$) *in vivo* fluorescence studies reported in [8], the AF bleaching rate values for healthy skin of 17 volunteers representing all six skin types (confirmed by a dermatologist) were determined with this technique. All the measurements were taken from the left forearm skin. The values related to volunteers of the same skin type agreed quite well, in most cases to within $\sim 20\%$. However, some specific differences were observed between the results obtained at two laser excitation wavelengths: 405 and 532 nm. At 405 nm excitation, the fast AF bleaching rate parameter τ_1 values within the 3–8 s interval were determined for all six skin types. The slow bleaching parameter τ_2 had values of 48–65 s for the first through third skin types, and 80–90 s for the fourth through sixth skin types. Generally, all six skin types responded similarly to 405 nm excitation.

At the 532 nm laser excitation, the slow bleaching rates τ_2 for the first, second, fourth, and fifth skin types also appeared to be similar (85–120 s), but substantially higher τ_2 values (250–350 s) were observed for the third skin type. The τ_1 values for the first through fifth skin types were within the 4–7 s interval, which is quite close to the fast AF bleaching rates determined during the violet laser excitation. Much weaker fluorescence from the sixth (hyperpigmented) skin type could be registered under the 532 nm laser excitation, and, what is most important, without any bleaching effects during irradiation. One can conclude that high melanin content in the epidermal layer stops the photobleaching process during the green 532 nm laser irradiation, but does not stop it during the violet 405 nm laser excitation.

Our single-spot measurements gave the mean τ values of the skin AF bleaching, integrated over the whole spot area, $\sim 4 \text{ mm}^2$. The new imaging technology opened the possibility to determine and display a better resolved distribution of these values across a much larger skin area, $\sim 4 \text{ cm}^2$. Figure 4 illustrates such distributions of the fast bleaching rates at the two laser excitation wavelengths for

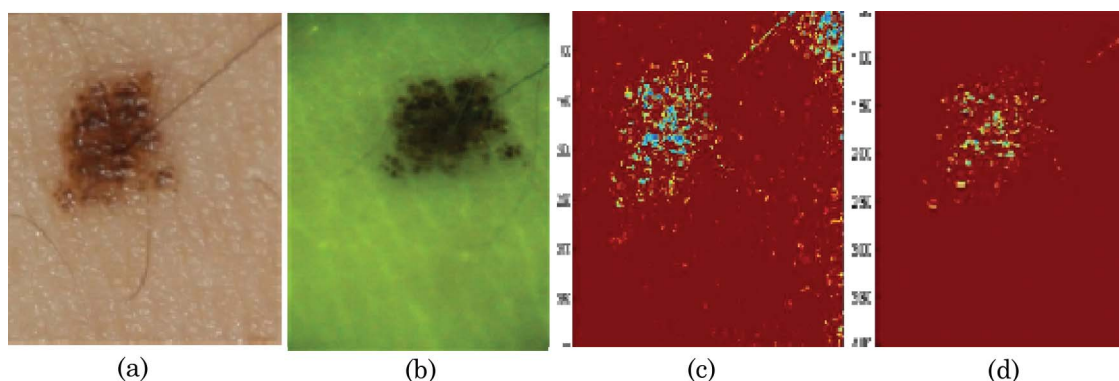


Fig. 7. (Color online) Images of a medium pigmented skin *nevi* at 405 nm laser irradiation: (a) ordinary photography, (b) filtered 405 nm laser excited AF image, (c) image of the fast AF bleaching rates (τ_1), (d) image of the slow AF bleaching rates (τ_2).

the fourth skin type. In both cases we observed some clusters or grains with a lower AF bleaching rate, i.e., higher τ_1 value than in the surrounding skin areas. Eventually, such grains are formed of some slower bleaching skin chromophores or their clusters.

B. Skin Pathology (*Nevi*): Conventional Photos and AF Bleaching Rate Images

If an ordinary photograph and image of AF bleaching rates taken at the skin pathology area are compared, more structured composition can be observed at the bleaching rate images, especially at 532 nm laser excitation. Figure 5 illustrates this for medium pigmented *nevi*; Fig. 6 illustrates this for hyperpigmented *nevi*. When the bleaching rate images related to the fast and slow rates are compared, we observed that more information is available for the fast or τ_1 images, and this seems to be true for both excitation wavelengths (Figs. 7 and 8). Nevertheless, some new details (eventually, pigment clusters) can also be found in the slowly bleached images.

C. AF Bleaching Rate Images of the Skin–Nail Interface

If different tissues are laser irradiated simultaneously, the AF bleaching rate images show new structural patterns also in the tissue interface regions. As an example, nail and skin areas are compared in Figs. 9 and 10 and reveal specific structures of the nail and the nail–skin interface under violet and green laser irradiation.

D. Spectral Features of the Laser-Excited AF at Different Bleaching Rate Spots

The hyperspectral imaging technology has been appropriated to obtain spatially resolved spectral information from the tissue surface spots with substantially different bleaching τ values. Figure 11(a) shows the 405 nm excited and spectrally filtered AF image at a skin *nevi* area; Fig. 11(b) shows the same image after processing, demonstrating the spectral differences of emission at the center of the *nevi* and around it. This image reveals some asymmetry of the *nevi* which looks nearly round in the fluorescence image (Fig. 11(a)). Even a more structured skin image has been obtained after processing with respect to the AF fast bleaching rates (Fig. 11(c)). Three spots of this image, one at the center of the *nevi* and two outside it but characterized by different τ_1 values, have been further analyzed spectrally (Fig. 12). At the center of the *nevi* the emission spectrum clearly differs from the two related to the spots outside the *nevi*. In spite of different τ_1 values at these two spots, both related emission spectra (the upper and the lower) look fairly similar. Our hyperspectral measurements at the 532 nm laser excitation did not show substantial differences between the spectra taken inside and outside of a medium-pigmented *nevi*, even if the bleaching rates at the particular image pixels were notably different. It is worth mentioning that the spectrally filtered gray-scale *nevi* AF image at the green 532 nm excitation seemed to provide more information

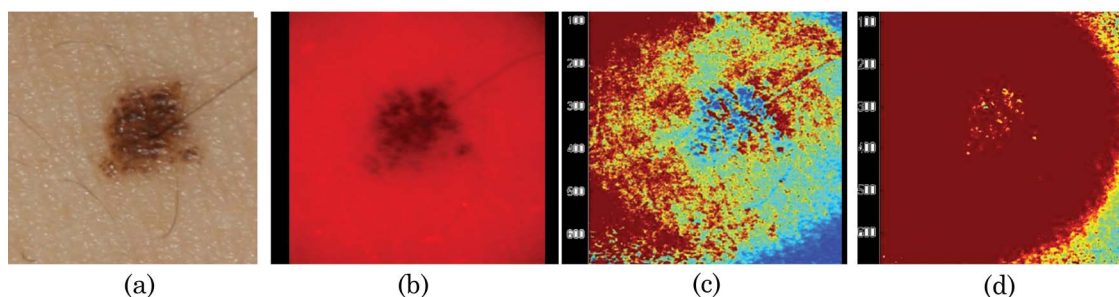


Fig. 8. (Color online) Images of a medium pigmented *nevi* at (a) 532 nm laser irradiation, (b) filtered fluorescence image at 532 nm laser excitation, (c) fluorescence bleaching τ_1 rate image, (d) 532 nm laser fluorescence bleaching τ_2 rate image.

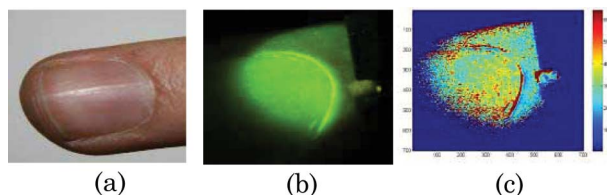


Fig. 9. (Color online) Images of a nail-skin interface: (a) photograph, (b) filtered fluorescence image at 405 nm excitation, (c) laser fluorescence fast bleaching rate image. The color scale represents τ_1 values in seconds in multiples of 10.

compared with that obtained at the violet 405 nm excitation (Fig. 11(b)).

4. Discussion

The major result of this work is the novel methodology for tissue surface structure studies and two experimental setups that implement this methodology. Imaging the AF bleaching rates could successfully complement the existing fluorescent techniques that are used for clinical assessment of skin and other disorders, e.g., cardiovascular [18,19] disease. Feasibility of the proposed methods and equipment has been confirmed by several clinical examples. In all the cases new structural tissue surface patterns, hidden in ordinary images, were revealed. However, these illustrations serve only to demonstrate the capabilities of the proposed technique. With regard to their clinical interpretation, serious further studies are needed. Only some general conclusions can be drawn from our initial results. For example, in the case of skin it seems that the fast AF bleaching images contain more clinical information if compared with the slow AF bleaching images. If the results at two laser excitation wavelengths are compared, it seems that AF bleaching at the green (532 nm) excitation might provide more information for dermatologists. The results could be related to a deeper penetration of the green 532 nm radiation under the skin surface, causing more efficient absorption and photobleaching of specific chromophores located at different skin depths.

An interesting feature of the hyperpigmented skin (sixth type) was observed: its AF bleached the same as all the other skin phototypes during 405 nm excitation but did not bleach at all at 532 nm excitation, the direct opposite of all the other skin phototypes which could be the result of high skin melanin content. One should note that, in all the illustrated cases

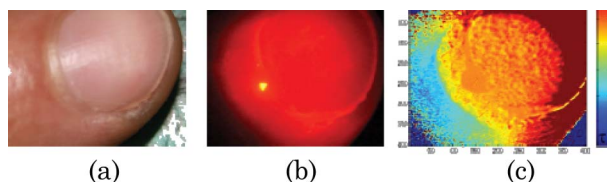


Fig. 10. (Color online) Images of a nail-skin interface: (a) photograph, (b) filtered fluorescence image at 532 nm excitation, (c) laser fluorescence fast bleaching rate image. The color scale represents τ_1 in seconds in multiples of 10.

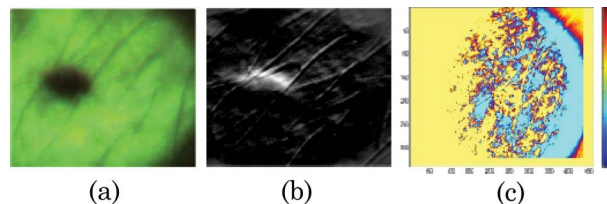


Fig. 11. (Color online) Images of a medium-pigmented skin *nevi* at 405 nm excitation: (a) filtered fluorescence image, (b) the same image after spectral processing (pixels with more significant spectral differences relative to the center of *nevi* are darker), (c) AF fast bleaching rate image. The color scale represents τ_1 values in seconds in multiples of 10.

with pigmented skin *nevi* (which contains more melanin than the surrounding tissue), the measured AF bleaching rate in the pathology location was slower. Eventually, melanin (and/or its compositions with other chromophores) could act as an agent slowing down the skin AF bleaching rate. This phenomenon certainly needs additional detailed studies.

With regard to our first hyperspectral imaging results, both excitation wavelengths could be useful for clinical diagnostics. In the case of medium-pigmented skin *nevi*, the AF spectrum at the center of pathology was clearly different from the spectra of surrounding healthy tissues at the violet 405 nm excitation, but no essential differences were observed at the green 532 nm excitation. On the other hand, the processed image that emphasized the relative spectral differences at the center and surroundings of this pathology seemed to be more structured at 532 nm excitation when compared with the 405 nm excitation.

Another aspect to discuss is laser safety. Although we did not exceed the commonly accepted laser skin exposure limits, it seems that even very weak laser irradiation ($\sim 1 \text{ mW/cm}^2$) could modify the skin fluorophore content, which is expressed as unevenly distributed AF bleaching. Skin recovery (regeneration of normal fluorophore proportion) after laser irradiation takes some time, and skin is probably more vulnerable during this recovery period with respect to infections, inflammations, or other disorders. To

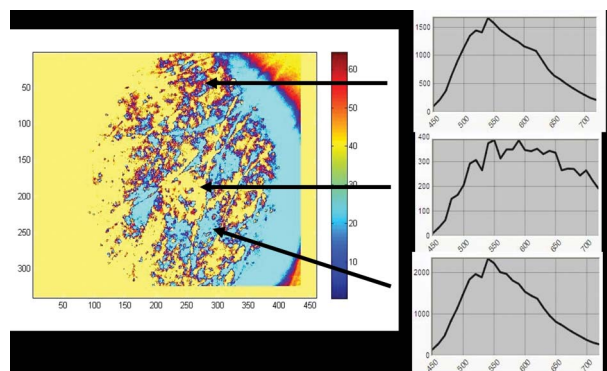


Fig. 12. (Color online) Emission spectra recorded at three different pixels of the 405 nm excited fast bleaching rate image of a skin *nevi*; see Fig. 11(c).

our knowledge, such cases have not been reported so far, but studies in this direction could provide new, useful information on skin response to laser irradiation.

5. Conclusions

To conclude, the newly developed technology appears to be well suited for advanced *in vivo* assessment of human skin and other tissues, including pathologic tissue, and could help to reveal the tissue surface structure details that are hidden in conventional images. Additional studies by use of this methodology would promote a better understanding of the photophysiological processes that take place in the human skin and other tissues.

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