Melanin Fluorescence Spectra by Step-wise Three Photon Excitation

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ABSTRACT

Melanin is the characteristic chromophore of human skin with various potential biological functions. Kerimo discovered enhanced melanin fluorescence by stepwise three-photon excitation in 2011. In this article, step-wise three-photon excited fluorescence (STPEF) spectrum between 450 nm -700 nm of melanin is reported. The melanin STPEF spectrum exhibited an exponential increase with wavelength. However, there was a probability of about 33% that another kind of step-wise multi-photon excited fluorescence (SMPEF) that peaks at 525 nm, shown by previous research, could also be generated using the same process. Using an excitation source at 920 nm as opposed to 830 nm increased the potential for generating SMPEF peaks at 525 nm. The SMPEF spectrum peaks at 525 nm photo-bleached faster than STPEF spectrum.

Keywords: Melanin, step-wise, two-photon excitation, three-photon excitation, multi-photon excitation, spectrum, fluorescence

1. INTRODUTION

Melanin is the characteristic chromophore of human skin with various potential biological functions, which include protection from solar radiation, antioxidant defense, and camouflage. Melanin is also involved in skin diseases such as malignant melanoma, an aggressive skin cancer with high metastatic potential. Despite its importance, melanin is poorly understood because it is an insoluble polymer without well-defined structure, which is difficult to isolate and study. [1]

Previous research has shown that stepwise two-photon excitation of melanin fluorescence can be generated by 800nm 120 fs-pulse laser with an emission spectrum that peaks at around 525nm. [2, 3] The process of stepwise two-photon excitation of melanin fluorescence is different from the generally known process of simultaneous two-photon excitation of fluorescence. The former is a two-step process via a real intermediate excited state energy level, whereas such a real intermediate state is missing in the latter case. Stepwise two-photon excitation needs only two- or more orders of magnitude lower excitation intensity to obtain the same population density of the fluorescent level as compared with simultaneous excitation. [3] Therefore, simultaneous two-photon excitation can only be generated using an expensive ultrafast laser, whereas stepwise two-photon excitation can be generated using a low cost continuous wave (CW) laser such as diode lasers. Although it is claimed that the melanin fluorescence reported is a two-photon excitation process, it is still unclear that whether the process here is a two-order process or higher-order process. In this article, we will define this process as step-wise multi-photon excited fluorescence (SMPEF).

Kerimo discovered enhanced melanin fluorescence by stepwise three-photon excitation. [4] This kind of fluorescence emits a much stronger signal than the previously reported SMPEF. However, that work did not include the emission spectrum. Here, we report our preliminary spectral results.

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2. MATERIALS AND METHODS

2.1 Samples

The eumelanin sample form Sepia was purchased from the Sigma Chemical Company. The black human hair and white human hair samples were from a 27 year-old man. The blonde human hair sample was from a 31-year-old man. All Sepia eumelanin samples and human hair samples were mounted inside culture dishes so that the air could be purged out with nitrogen in order to reduce the photo-bleaching effect. The rat skin and hair samples were from the skin of a laboratory rat sacrificed in unrelated research and the zebra fish samples were 7-day-old larvae.

2.2 Fluorescence Measurement

The equipment for the measurement of step-wise three-photon excited fluorescence (STPEF) spectra is depicted in Figure 1. Excitation was performed with a Tsunami Ti: Sapphire laser (repetition rate 80MHz, excitation pulse duration, 100fs, center wavelength is tunable from 750 nm to 1000nm). The laser was switched between pulsed mode and continuous wave (CW) mode for comparison between spectra. The scanner was a polygon mirror which produced a line-scan at a 6 kHz line rate, suitable for coupling into the spectrometer. The dichroic mirror passed the laser and reflected fluorescence signals into a grating (Princeton Instruments 150 g/mm 500 nm Blaze). The detection system was a charge-coupled device (CCD) camera (SPOT RT900). Here, we used a CCD instead of a PMT due to the fact that the CCD has a longer working range at the near-infrared region and has many more detection channels. Although the sensitivity of the CCD is lower than the PMT, it is good for this application as the STPEF signal of melanin is very strong. The spectral response of the system was calibrated by using a high quality tungsten lamp (USHIO JC12V-50W). We assumed that the spectrum of the tungsten lam followed Plank's distribution. The data above 700nm was discarded as the sensitivity of the system was low at that range.

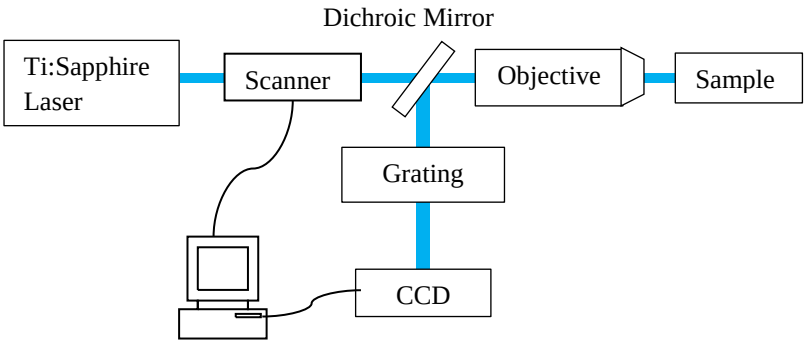


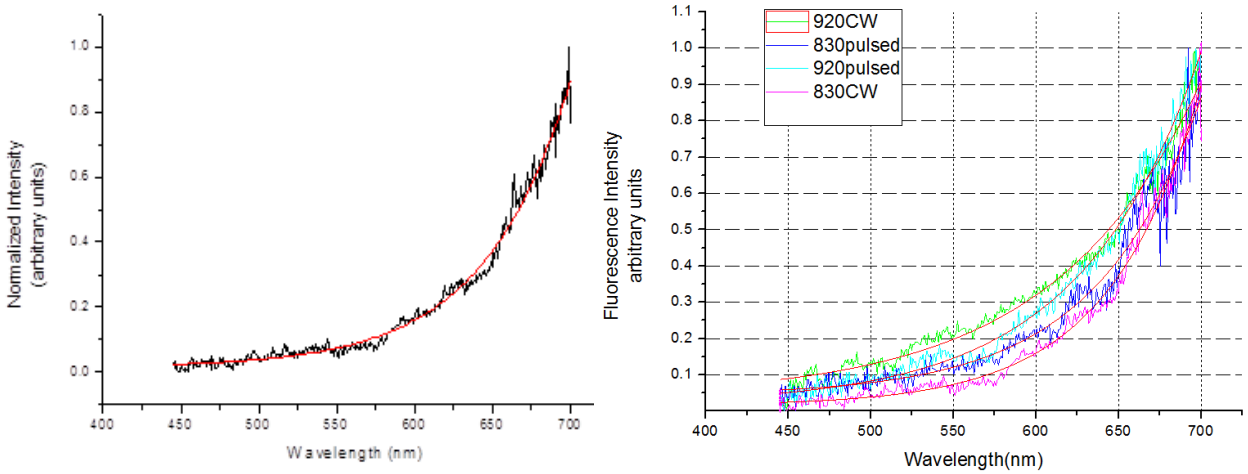
Figure 1: Experimental setup for spectral measurements of melanin by step-wise three photon excitation

3. RESULTS AND DISCUSSION

3.1 Fluorescence Spectra of Sepia Melanin

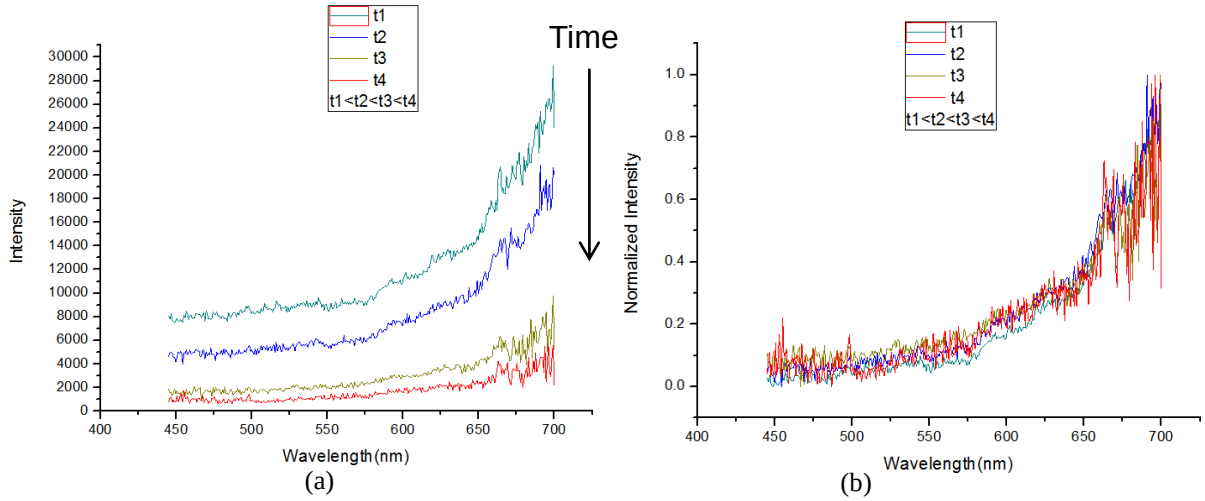
The STPEF spectrum of sepia melanin is shown in Figure 2a. The spectrum followed an exponential increase with wavelength. The excitation source dependence of sepia melanin STPEF spectrum was also studied. The shapes of the fluorescence spectra remained exponentially distributed under four different excitation source: 830 nm CW, 830 nm pulsed, 920 nm CW, 920 nm pulsed.

The time dependence of STPEF spectra of sepia melanin was also examined (Figure 3). As time increased ($t_{red} > t_{yellow} > t_{blue} > t_{green}$), the intensity of the fluorescence signal decreased (Figure 3a), but the spectra remained the same shape when normalized (Figure 3b). This indicates that we were not creating any new emissive components as the melanin was photo-bleached.



(a) (b)

Figure 2a, STPEF spectrum of sepia melanin (black). Red curve is the exponential fit of the spectrum. $\lambda_{exc} =$ 830 nm CW. Figure 2b, Excitation source dependence of sepia melanin TPEF spectrum. The excitation source dependence of sepia melanin STPEF spectrum was also studied. The shapes of the fluorescence spectra remained exponential distribution under different excitation source.



(a) (b)

Figure 3, Time dependence of TPEF spectra of sepia melanin. $t_{red} > t_{yellow} > t_{blue} > t_{green}$. As time increased, the intensity of fluorescence signal decreased (Figure 3a). But the spectra remained the same shape when normalized (Figure 3b). $\lambda_{exc} = 830\, nm\, CW$.

Although most spectra followed an exponential distribution with wavelength (blue curve in Figure 4a), there was a substantial minority (35.71%) that had a different shape (red curve in Figure 4a). In Figure 4a, we compare typical curves for the majority (blue) and minority emission curves we have observed in this work. It is clear that there is an additional peak at about 525 nm for the minority of our observations. When we subtract the two curves we obtain Figure 4b. The spectrum in Figure 4b peaks at around 525 nm, and is very similar to the SMPEF spectrum of melanin reported previously. [2, 3] Therefore, we believe that the red curve in Figure 4a is actually a mixed spectrum of SMPEF spectrum and STPEF spectrum. But it is clear that the SMPEF spectrum is just a small component of the integrated fluorescence signal.

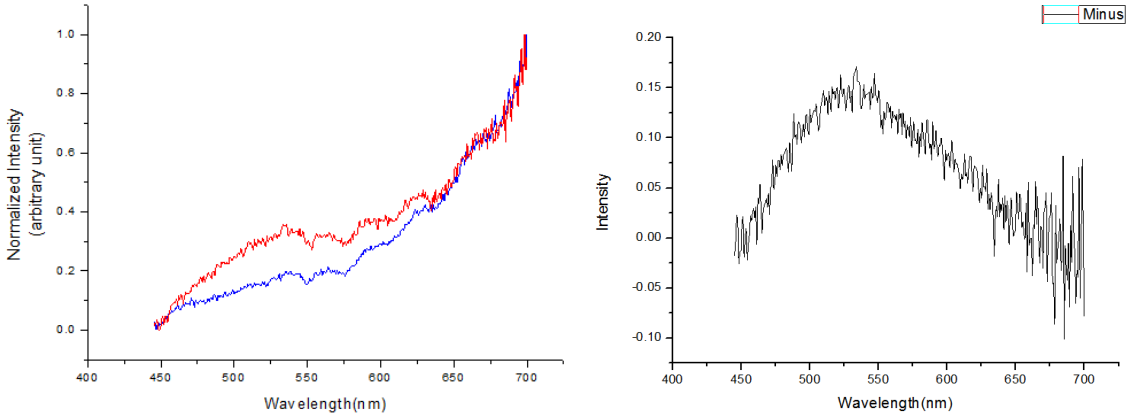


Figure 4a, TPEF spectrum (blue) and mixed spectrum (red). $\lambda_{exc} = 830$ nm CW. Figure 4b, mixed spectrum subtracted by TPEF spectrum.

3.2 Fluorescence Spectra of Melanin in Tissue

Fluorescence-spectra of melanin in different tissue are shown in Table 1. The first column contains the name of the samples. The second column shows the STPEF spectra from different samples. The third shows the mixed spectra of both SMPEF spectra and STPEF spectra. In human hair, different colors of hair were also compared. Yellow curves are melanin fluorescence spectra from blonde hair; black curves are melanin fluorescence spectra from black hair; the green curve is the melanin fluorescence spectrum from white hair.

3.3 Mixed Spectra Decay to STPEF Spectra with Time

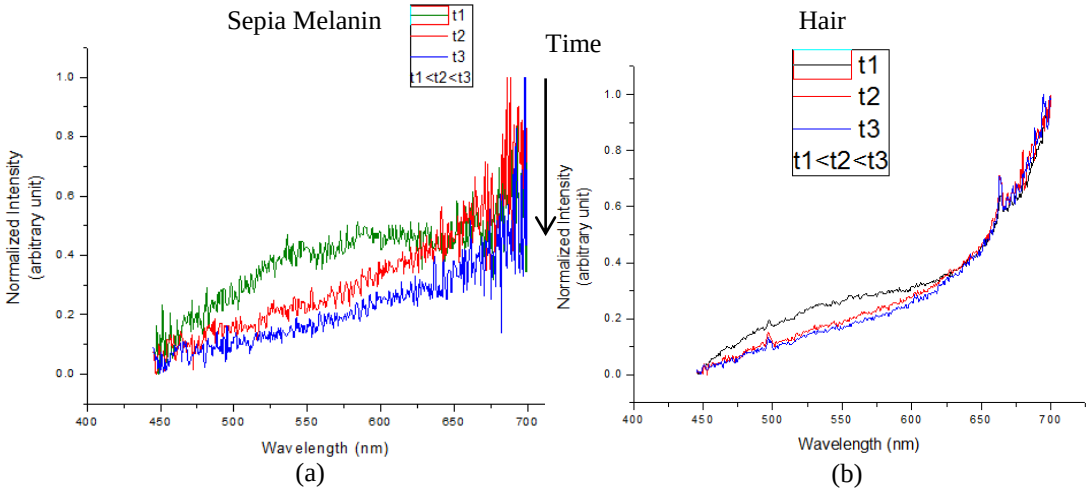
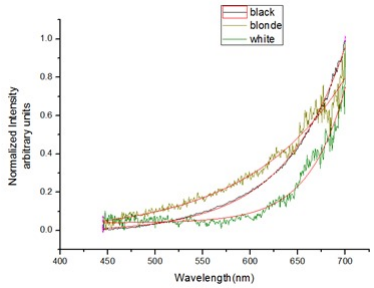
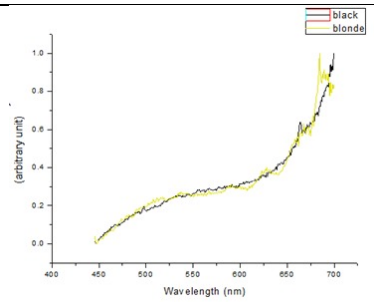
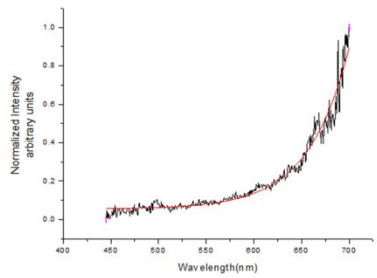
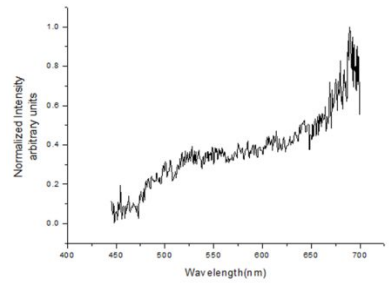
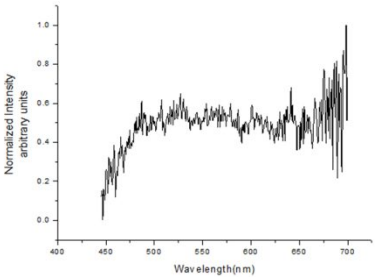
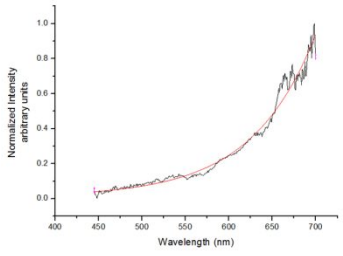
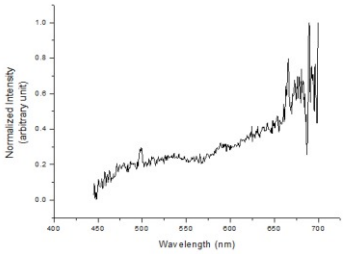


Figure 5a, Time dependence of mixed spectra of sepia melanin. $t_{green} > t_{red} > t_{blue}$. $\lambda_{exc} = 920$ nm CW. Figure 5b, Time dependence of mixed spectra of hair melanin. $t_{black} > t_{red} > t_{blue}$. $\lambda_{exc} = 830$ nm CW.

We also noticed that mixed spectra decayed to STPEF spectra at long times (Figure 5). This decay indicates that the SMPEF spectrum photo-bleached faster than STPEF spectrum.

Table 1, Fluorescence spectra of melanin in tissue

Sample	STPEF spectra	Mixed spectra	Percentage of Mixed Spectra
Human Hair	 $\lambda_{exc} = 920\ nm\ CW$	 $\lambda_{exc} = 920\ nm\ CW$	32.74%
Rat Hair	 $\lambda_{exc} = 920\ nm\ CW$	 $\lambda_{exc} = 920\ nm\ pulsed$	38.46%
Rat Skin		 $\lambda_{exc} = 920\ nm\ pulsed$	100%
Iris of Zebra Fish	 $\lambda_{exc} = 830\ nm\ CW$	 $\lambda_{exc} = 830\ nm\ CW$	73.91%

3.4 Probabilities of Generation of Mixed Spectra

Excitation source dependence of the probabilities of generation of mixed spectra from sepia melanin and human hair melanin were examined. Figure 6 shows that the probabilities of generation of mixed spectra did not change significantly

due to excitation laser mode (~ 33%), but the probabilities of generation of mixed spectra was much higher when the excitation laser was 920 nm (50% in sepia, 39.34% in hair) than that of 830 nm (25% in both sepia and human hair).

4. CONCLUSION

The spectra of STPEF followed exponential distribution with wavelength. There is a probability of about 33% that previously shown SMPEF can also be generated using the same process. As excitation source at 920nm has a higher chance of generating SMPEF peaks at 525 nm than source at 830nm, SMPEF spectra photo-bleach faster than STPEF spectra.

Further research will be focused on exploring the STPEF spectrum peak above 700 nm. The photo-bleaching effect of STPEF needs to be studied. Whether the SMPEF is a second-order or a higher-order process will be determined.

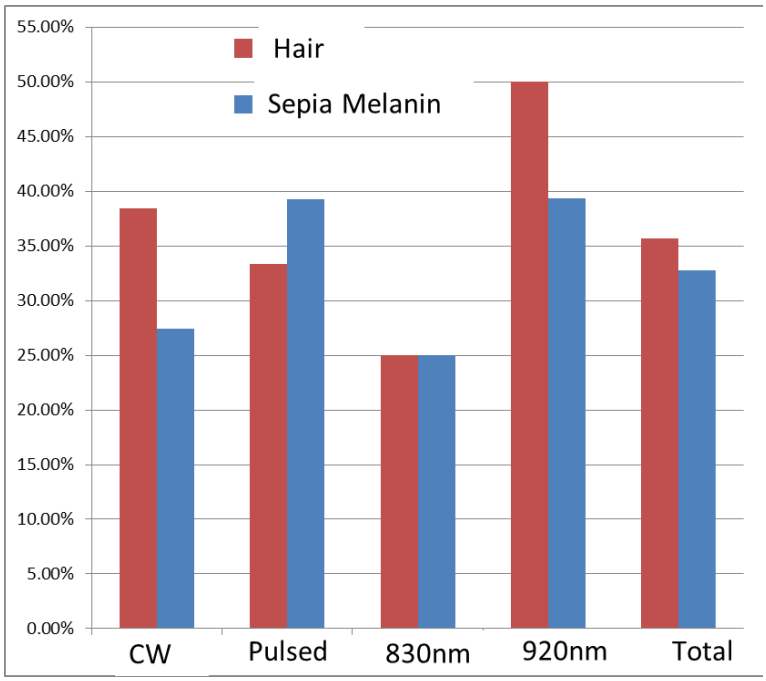


Figure 6, Probabilities of generation of mixed spectra – excitation source dependence

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