Three-photon fluorescence imaging of melanin with a dual-wedge confocal scanning system

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ABSTRACT

Confocal microscopy can be used as a practical tool in non-invasive applications in medical diagnostics and evaluation. In particular, it is being used for the early detection of skin cancer to identify pathological cellular components and, potentially, replace conventional biopsies. The detection of melanin and its spatial location and distribution plays a crucial role in the detection and evaluation of skin cancer. Our previous work has shown that the visible emission from melanin is strong and can be easily observed with a near-infrared CW laser using low power. This is due to a unique step-wise, (SW) three-photon excitation of melanin. This paper shows that the same SW, 3-photon fluorescence can also be achieved with an inexpensive, continuous-wave laser using a dual-prism scanning system. This demonstrates that the technology could be integrated into a portable confocal microscope for clinical applications. The results presented here are in agreement with images obtained with the larger and more expensive femtosecond laser system used earlier.

Keywords: Confocal microscopy, Melanin, Fluorescence, Risley prism

1. INTRODUCTION

Confocal microscopy, invented first in the early 1960's [1], has had many improvements and is close to becoming a preliminary diagnostic tool [2-5]. As can be easily observed even without the use of any optical equipment, lesions in general, and cancerous lesions in particular, appear darker than their surrounding tissue. The reason for that is the higher local production and concentration of melanin in the tissue [6-7]. Thus, specific detection of melanin is becoming more desirable since it can potentially reveal more information about the nature of the lesion. Indeed, previous studies showed that melanin plays a very crucial role in the diagnosis of cancerous lesions in-vivo [8]. In addition, the degradation of melanin over time, due to aging or light exposure, is important since it could be associated with the development of malignant melanoma or skin cancer [9]. Confocal imaging studies performed on patients with skin types 1-6 revealed that melanin demonstrates high reflection of infra-red light. Darker skin types showed higher contrast in confocal images due to higher reflection from melanin [10]. This high reflection of infra-red light by melanin, however, complicates its detection or identification when using confocal microscopy. Differentiating the reflected IR from melanin rather than from other scattering components in the tissue isn't a simple task. A recent study has shown that melanin can be imaged with high specificity using a unique stepwise three-photon fluorescence excitation process [11]. A combination of reflectance and step-wise three-photon fluorescence overcomes this problem by having the ability to specifically highlight, differentiate and locate the melanin from other high-reflectance materials. The earlier work used a femtoseond laser as is typical from multi-photon excitation, but also showed that the stepwise nature of the excitation allowed the use of CW laser source. In this work we demonstrate the combination of confocal reflectance and step-wise fluorescence of melanin in a portable dual-wedge scanning system.

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2. METHODS

2.1 Samples

We used pure samples of melanin from Sepia (M2649), purchased from the Sigma Chemical Company. It is in the form of dry black powder, which was spread on a R3L3S1N - Negative 1951 USAF Test Target purchased from Thorlabs. The powder was then held in place by a number one microscope cover-slip. The use of the USAF target as a background substrate served the purpose of assuring good registration of the location of the melanin particles while switching modalities. However, a unique pattern substrate is not necessary for the experiment. Individual granules of melanin could be observed. They ranged from less than a micrometer to tens of micrometer in size and were mostly spherical or oval in shape. The other sample that was studied consisted of strands of black human hair, about 50 micrometer in diameter, mounted directly on an EIA Gray-scale Pattern Slide from Edmund Optics. The purpose of the grayscale pattern was to provide background grid pattern and assist finding the focal location with respect to the hair sample, which had a wavy shape and was getting in and out of focus.

2.2 Microscope

A scanning confocal microscope that was made in-house was used to image the samples. The system used a M-x20 0.4 NA Newport objective, a custom-made set of cogs that spin the Risley-Prism setup to produce the scanning pattern, and a telescope lens set to assure correct projection of the image and pupil planes along the optical axis. The complete description of the system can be found elsewhere [12-13]. The optical layout of the dual-wedge scanner with the three-photon microscopy capability is shown in Fig.1. The new addition starts with the dichroic mirror, which transmits wavelengths longer than ~750 nm and reflects shorter ones. The source laser operates at 839 nm. Therefore, the use of the dichroic mirror allows the system to operate in a dual-modality mode, in which confocal reflectance and three-photon excited fluorescence can be obtained simultaneously.

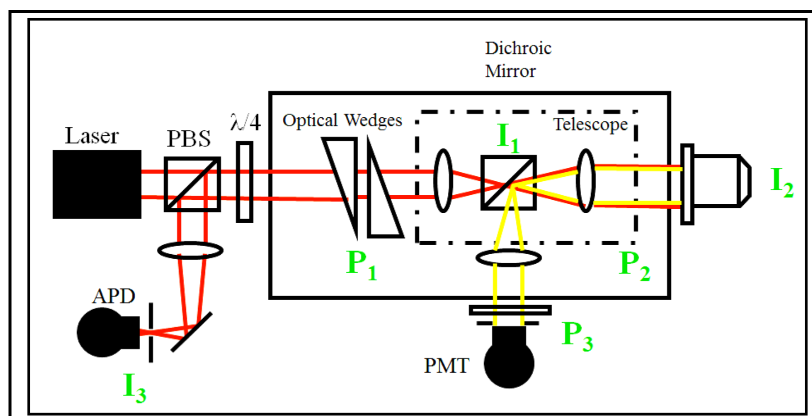

Fig 1: Optical layout for dual-wedge scanning confocal reflectance & three-photon fluorescence microscope

The PMT used was a Hamamatsu 3907-03, which displays a fairly high sensitivity even into the NIR region. The high sensitivity of the PMT in the NIR region can be problematic for imaging the fluorescence. Even a small fraction of the laser light, reflected from the dichroic to the PMT, could be more intense than the three-photon fluorescence. This would have limited the specificity of the PMT signal to the three-photon fluorescence. To overcome this problem, a 800nm short-pass filter was added in front of the PMT. The filter was verified to be robust enough to block the laser source at the beginning of the optical path, where the intensity is at its highest. This process ensured no reflection would leak into the PMT. Therefore any image captured by the PMT will consist of only of the fluorescence signal. In front of the PMT, a 1mm in diameter pinhole was also added. The purpose of the very wide pinhole was to limit the amount of light, and

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hence noise, at the PMT side, rather than to provide confocality. The location of the equipment, which was added to our previously existing setup, was chosen to be in between the two telescope lenses, in a pupil plane. This location is optimum for collecting the fluorescence signal where it serves a dual purpose: 1. The loss of fluorescence signal is minimal. 2. It is before the beam de-scans, so it it is stationary in the plane of the aperture. This allows the use of smaller aperture that helps preventing unwanted light from entering the PMT and interfering with the measurement.

3. RESULTS

The laser power at the sample was measured to be ~0.5mW. This power is enough to activate the melanin particles in order to see the enhanced melanin emission [11]. Fig.2 shows the images obtained from both confocal reflectance and three-photon fluorescence modalities, as well as the merged image of the two modalities (Fig.2(a), 2(b), and 2(c) respectively). It can be seen from the images that even in confocal reflectance mode, pure melanin strongly reflects the NIR light. In Fig.2(b) the background information is completely suppressed and only fluorescence from specific areas containing melanin are observed. From the merged image it can be seen that the locations that exhibit high three-photon emission are perfectly registered to the location of the melanin particles.

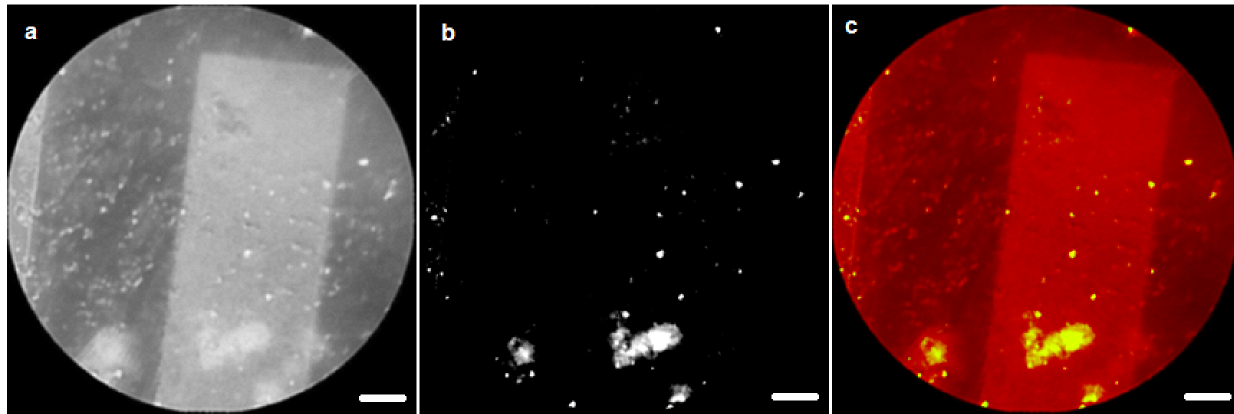

Fig 2: Images of enhanced emission of Sepia melanin in atmosphere. (a) confocal reflectance image. (b) three-photon image. (c) merged image. Scale bar is 10um.

The suppression of all background information when switching between confocal reflectance and three-photon fluorescence mode suggests that the origin of the signal is indeed the step-wise fluorescence process. This background suppression was only possible after adding the filter in front of the PMT. Prior to having the additional filter, the image included the USAF target. This resulted from the very small amount of reflected IR light, that entered the PMT. This image, provided the same information as confocal reflectance, however without the sectioning ability. That image was also lacking the needed specificity to melanin. Similarly, Fig.3 displays a series of images in the same order as in Fig.2, but of a dark human hair instead of melanin particles. In this case as well, the location of the fluorescence is perfectly aligned with the location of the hair sample, and no signal was seen elsewhere, where melanin is not present. In order to better demonstrate the fluorescence using the hair sample, the confocal image, Fig.3(a), and the three-photon excitation emission Fig.3(b) are slightly misplaced along the optical axis. By focusing the confocal reflectance on the substrate the hair appears darker than the background. The fluorescence image remained focused on the hair. Therefore, when merging the confocal with the excited fluorescence images, the fluorescence sites will have a better contrast and help demonstrate the phenomenon. As can be seen from Fig.2 and Fig.3, both in the case of pure melanin particles from Sepia and in the case of melanin from the human hair sample, not all areas that appear to have melanin present actually demonstrate three-photon fluorescence. This can be explained by Equation 1[14], which describes, for the general case, the power of an n-photons excitation fluorescence process as a function of the input continuous-wave laser power, the lifetimes of each state τ_j and the absorption cross section σ_i .

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$$P^{nF}=\frac{\partial\cdot\left(\prod_{i=1}^{n}\sigma_i\prod_{j=1}^{n}\tau_j\cdot\left\langle P_{laser}\right\rangle^n\right)}{\partial t}. \tag{1}$$

In our case, n=3; the excitation process is a non-linear process of third order. The excitation, although step-wise, will occur to a much greater extent inside the volume of the point of focus than outside of it. The emphasis on step-wise is to distinguish this process from a three-photon fluorescence process where only virtual intermediate states exist. The probability of such a process is orders of magnitude smaller and very likely to be undetectable.

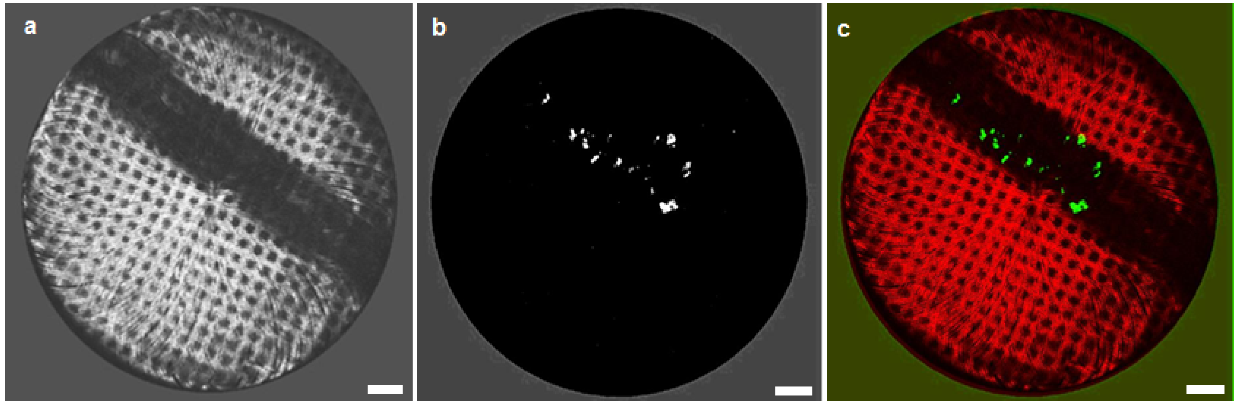

Fig 3: Images of a dark human hair in atmosphere. (a) confocal reflectance image. (b) three-photon image. (c) merged image. Scale bar is 10um.

Fig.4 shows a simulated model of the power distribution along beam profile for both cases. The model is build on the a theoretical "Gaussian beam" model as described by Equation 2 [15-16].

$$I(\rho,z)=\frac{2P}{\pi\cdot W^2(z)}\cdot e^{-2\frac{\rho}{W^2(z)}}. \tag{2}$$

Where P is the total power carried by the beam, W(z) is the beam width, and ρ is the beam radius. As can be seen in Fig.4, in the case of three photon excitation, a significantly higher emission of fluorescence occurs inside the focal point compared to outside of it. This will result with the ability to perform optical sectioning without the need for a pinhole. In Fig.2, the pure melanin case, some of the melanin particles stuck to the cover-slip, while others remained on the surface of the USAF target. The gap between the two surfaces can very easily exceed 20um, which is approximately the length of the excited volume along the focal axis. Therefore, some regions will remain outside of this focal volume, where the three-photon fluorescence excitation is generated. These regions correspond with particles that did not appear to be emitting any fluorescence when switching modalities.

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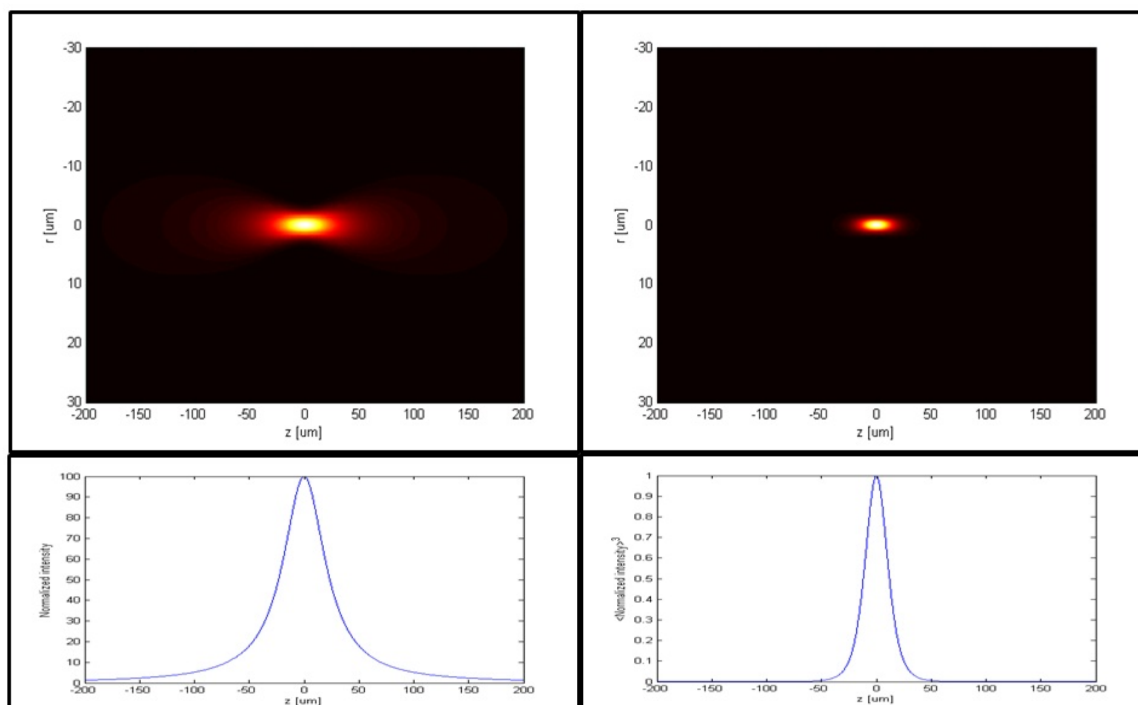

Fig 4: The beam profile of a linear (left column) vs. third-order process (right column).

In the human hair case of Fig.3, the sample was not held under a cover-slip. In this case, however, changes along the focal axis, which occurred due to the wavy nature of the hair will have the same effect. In addition, the melanin distribution inside the focused region, is in the form of spot shaped clusters, as typical to the distribution of melanin in hair.

4. DISCUSSION

The stepwise three-photon fluorescence in melanin, which was observed in our lab earlier using a multiphoton microscope with a femto-second pulsed laser, was observed with a much less expensive and more compact setup using a dual wedge confocal scanning system and a continuous wave laser. The results show excellent agreement with those obtained earlier using the pulsed laser. Our observation of fluorescence using the dual-wedge microscope demonstrates that a low-cost CW laser and a simple scanner can be used to detect melanin while simultaneously imaging structure with a references confocal microscope. In a skin tissue the absorption of near infra-red light is very limited and the emission of 3-photon fluorescence, with a peak intensity at around 450nm, will unveil the location of melanin. This combination of imaging modalities can be integrated into a hand-held type instrument for early skin cancer diagnosis.

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