ANALYSIS STUDY AND IMAGING ON STEPWISE MULTI-PHOTON ACTIVATION FLUORESCENCE OF MELANIN

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Abstract

In this thesis, the author’s work is divided into two aspects: multi-modal microscopy and the stepwise multi-photon activated fluorescence (SMPAF) of melanin.

A multimodal microscope provides a researcher with the ability to capture images with multiple sources of contrast. The Keck 3D fusion multi-modal microscope (3DFM) in the Optical Science Laboratory (OSL) at Northeastern University allows us to image samples with multiple different modalities on the same stage. During the course of this thesis, we upgraded the whole control system and part of the optical layout. Another multimodal microscope (the Target System) is also designed for precise positioning.

The research on the stepwise multi-photon activated fluorescence (SMPAF) of melanin using KECK 3DFM is also reported in the thesis. We have shown previously that melanin goes through a step-wise multi-photon absorption process after the fluorescence has been activated with high laser intensity. The SMPAF of melanin has been observed to require less instantaneous laser power than what would be expected from a non-linear optical process, which can be activated by a continuous-wave (CW) mode near infrared (NIR) laser. Therefore, the SMPAF has potential applications for a low-cost method of detecting melanin. We conducted the statistical studies of melanin SMPAF to understand the activation process and photo-bleaching effect. In addition, the application of melanin SMPAF imaging for purpose of detecting melanin inside human skin in vivo has been demonstrated.
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Introduction

1.1 Skin cancer, Melanoma and Melanin

Skin cancer is the most common type of cancer in fair-skinned populations in many parts of the world (1, 2). As the incidence of skin cancer is increasing at an alarming rate, it is one of the greatest threats to public health (2, 3). Skin cancers are mainly divided into melanoma, and non-melanoma skin cancers (NMSCs), the NMSCs including basal and squamous cell carcinomas (BCC and SCC, respectively). Melanoma is responsible for most of the cancer related mortalities, and NMSCs are typically described as having a more benign course with locally aggressive features. Nevertheless, they represent “the most common type” of cancer in humans and they can result in significant disfigurement, leading to adverse physical and psychological consequences for the affected patients (4).

Melanoma is a unique and highly aggressive type of cancer that occurs more frequently with increasing age and often with a significant contribution of environmental factors to its etiology (5). It is estimated that 132,000 new cases of melanoma occur worldwide each year (6). Incidence rates are at least 16 times greater in Caucasians than African Americans and 10 times greater than Hispanics (7). The World Health Organization (WHO) estimates that as many as 65,161 people a year worldwide die from malignant skin cancer, approximately 48,000 of whom are registered(6, 8). Melanoma represents only about 3% of all skin cancers in the U.S., but it accounts for about 75% of all skin cancer deaths(9). The American Academy of Dermatology (AAD) estimated that in 2009, there will be about 121,840 new
melanoma cases in the U.S. with 8650 deaths(8).

Melanin is a nearly ubiquitous pigment in biology and largely responsible for the color of skin, hair and eyes (10, 11). Melanin can be divided into two main classes: Eumelanin, a black-dark-brown insoluble material found in black hair and in the retina of the eye, and Pheomelanin, a yellow to reddish-brown alkali-soluble material found in red hair and red feathers(12). Both types of melanin are important tissue chromospheres. The melanin absorbs harmful Ultraviolet Radiation (UVR) and more than 99.9% of the absorbed UVR as heat which makes it an excellent photoprotectant (1, 6, 12). However, the exact role of melanin’s activity is not known, as it has also been known to produce free radicals in response to the UV radiation, which may be toxic. In addition, the pigments appear to exhibit many properties such as photoconductivity that appear to have no known biological significance. However, it is clear the optical properties of melanin play an important role in its function (13, 14).

Melanin is related to many skin diseases. e.g. malignant melanoma, the most aggressive skin cancer; vitiligo, a disease characterized by the loss of melanin pigment (15); melasma, an acquired brown hypermelanosis of the face (16); solar lentigines and ephelides, benign pigmented spots which are associated with an increased risk of skin cancer (9, 17, 18); and nevus of ota, a syndrome consisting of a grayish-blue, macular discoloration affecting the sclera of an eye and the ipsilateral facial skin in the area of the distribution of the corresponding trigeminal nerve (19).
1.2 Melanin fluorescence

The auto-fluorescence of human skin tissue are usually excited by one-photon absorption in the UV-A region, with expected fluorescence of melanin in the spectral region of 450nm-650nm(20). However, this melanin fluorescence is ultra-weak, and in the auto-fluorescence of human skin tissue it is completely hidden by the emission from the main endogenous fluorophores(21). This absence of melanin fluorescence is a disadvantage because melanin fluorescence may be a useful source of information about the status of pigmented skin(11).

Han et al. attempt to obtain melanin fluorescence in complex media is by conventional one-photon excitation in the NIR-region around 800 nm (22-24). No endogenous skin tissue fluorophores except melanin show any absorption at this wavelength.

Another attempt to overcome the mentioned disadvantage is aimed at uncovering the hidden melanin fluorescence of skin tissue by a special fluorescence excitation technique, based on two-photon absorption from laser radiation in the 800 nm region. This is caused by a special non-linear optical effect called simultaneous two-photon absorption. The intensity of such excited fluorescence is comparably weak. By contrast, melanin shows absorption in the 800 nm range; upon irradiation with 800-nm laser pulses, it absorbs two photons in a stepwise process via an intermediate excited electronic state (21, 25). These first investigations used femtosecond laser pulses.

An important enhancement in measuring the melanin fluorescence from skin tissue was recently
using nanosecond pulses instead of femtosecond pulses(26). Assuming the same number of photons per pulse, the photon flux in the nanosecond pulse is much lower than in the femtosecond pulse. Therefore, for simultaneous two-photon absorption of fluorescence, a couple of photons must be presented instantly, whereas melanin after absorption of a first photon ‘can wait’ picoseconds or even longer for the photon of the second absorption step.

A fluorescence technique directed at these melanin fluorescence specifics may be used to distinguish benign melanoma and melanocytic lesions in either histopathologic specimens or in vivo in the skin (25).

1.3 Stepwise Multi-Photon Activation Fluorescence (SMPAF)

Kerimo et al. in optical science laboratory of Northeastern University discovered enhanced melanin fluorescence by stepwise three-photon excitation(13). He observed that the fluorescence of eumelanin (from Sepia and black human hair) was activated and enhanced by almost three orders of magnitude by exposure to near-infrared radiation. The third-order dependence is explained by a step-wise excited-state absorption process since the same third-order dependence was present when either continuous wave or femtosecond pulsed laser, with similar average-power levels, was used. The author has defined this process as stepwise multi-photon activated fluorescence (SMPAF) to distinguish the process discovered by Kerimo from the stepwise multi-photon excited fluorescence (SMPEF) observed by Teuchner and Hoffmann, which does not require the activation step(14, 21).
Lai reports the spectra of this SMPAF in the region of 450nm-700nm(27). The spectra of STPAF followed exponential distribution with wavelength. As excitation source at 920 nm has a higher chance of generating SMPEF peaks at 525 nm than source at 830 nm, SMPEF spectra photo-bleach faster than SMPAF spectra. Mega also shows that the melanin SMPAF can also be achieved with an inexpensive, continuous-wave laser using a dual-prism scanning system in year 2012 (28).

Lai demonstrated that the melanin SMPAF reveals a broad spectrum extending from the visible spectra to the NIR and has potential application for a low-cost, reliable method of detecting melanin(14). SMPAF images of melanin in mouse hair and skin are compared with conventional multi-photon fluorescence microscopy and confocal reflectance microscopy (CRM). Also, a photo-bleaching effect analysis is reported in his paper.

Lai conducted further research using even higher laser intensity for the activation, and have shown the possibility of observing power dependence other than third-order(29). This article discusses the possible energy states of Sepia melanin by studying the power dependence curves of the step-wise multi-photon activated fluorescence signal.

1.4 Thesis structure

In this thesis, the author’s work is divided into two aspects: mutli-modal microscopy and the stepwise multi-photon activated fluorescence (SMPAF) of melanin. In Chapter 2, the design and implementation of KECK 3DFM 2.0 system and target system are presented. Chapter 3 goes into the
details of statistical studies for SMPAF of melanin to study the activation processing and the photo-bleaching effect of the fluorescence. Chapter 4 is the related application of melanin SMPAF, where In-vivo SMPAF imaging on Human Skin and activation threshold measurement of SMPAF. Chapter 5 concludes the thesis with a brief discussion of the multi-modal microscopy and SMPAF of melanin. The future work that needs to be assessed is also shown in this chapter.
2 “KECK” 3D Multimodal Microscopy

2.1 Introduction

A multimodal microscope provides a researcher with the ability to capture images with multiple sources of contrast on a single platform to understand any biological system (30). While each source of contrast provides unique advantages, a multimodal microscope can integrate benefits of each modality. By overlaying the different modalities’ images obtained simultaneously from the multimodal microscope, a more comprehensive view of different tissues can be obtained.

The Keck 3D fusion multi-modal microscope (3DFM) in the Optical Science Laboratory (OSL) at Northeastern University was first built by Daniel J. Townsend in 2004 (30, 31). The 3DFM allows us to image samples with multiple modalities on the same stage. The modalities on the 3DFM include: Brightfield Microscopy, Differential Interference Contrast Microscopy (DIC), Epifluorescence Microscopy (EFM), Confocal Reflectance Microscopy (CRM), Confocal Fluorescence Microscopy (CFM), Multi-Photon Fluorescence Microscopy (MPFM), and Second Harmonic Generation (SHG). An imaging spectrometer is integrated in the 3DFM for spectrum measurement.

The KECK 3DFM underwent a major upgrade in the year 2013. The upgrade to KECK 3DFM 2.0 was led by the Zhenhua Lai and assisted by Stephen Karasek, James Mclean and the author. The whole control system and part of the optical layout were redesigned. The performance of the second generation 3DFM has been significantly improved after the upgrade. The design of the 3DFM 2.0 is introduced in
this chapter. The author in this work is response for the control system design and electrical instrument installation.

2.2 Microscopy Background

This section gives a brief background to each mode of microscopy used in updating the KECK 3DFM 2.0. Only the modality related to the author’s work will be introduced here. The history and basic concepts of each microscope will be shown.

2.2.1 Confocal Reflectance Microscopy (CRM)

Marvin Minsky invented the confocal microscope in the year of 1957 (32). Confocal reflectance microscopy offers several advantages over conventional widefield optical microscopy with an increased signal-to-noise ratio, including better imaging through rejection of information obtained from regions removed from the focal plane, the ability to collect serial optical sections from thick specimens, and the ability to regulate the depth of field.

The key to confocal microscopy is the use of spatial filtering to eliminate out-of-focus light from specimens that are thicker than the focal plane as shown in Figure 2-1. In most confocal microscopes the aim is to illuminate with light that is focused to the very smallest spot possible in the plane of focus(33). The CRM detects light that is backscattered into the objective lens. The illumination light is collimated by the lens, reflected by the beam splitter, and then focused to the sample through the objective. The
backscattered light is collected by the same objective, passed through the beam splitter, and then recorded by the detector. Out-of-focus light (red dash dot line) is rejected by the aperture on the detector.

Figure 2-1 Layout of a conventional confocal reflectance microscope (CRM)

A switchable quart-wave plate is placed in the layout between the objective and the beam splitter if the light source is polarized; meanwhile, the beam splitter should be a polarization beam splitter, i.e. the beam splitter reflects light with one polarization and passes light with perpendicular polarization. The quarter-wave plate turns the backscattered light reflected from the sample into the polarization that is perpendicular to the input light. In this way, the signal reaches the detector is maximized.
2.2.2 Confocal Fluorescence Microscopy (CFM)

The laser beam is focused on the transparent specimen, and fluorescence light is emitted directly back through the specimen using the same optical path while passing through an emission filter and a small aperture (pinhole) before reaching the detector(34). Only light from the focal plane is detected because the light coming from other planes cannot pass through the aperture (Figure 2-2).

The difference between the confocal reflectance (CRM) and confocal fluorescence microscope (CFM) is that the CFM uses the dichroic mirror while CRM uses the beam splitter for distinguish the...
input light and reflectance light. The Dichroic mirrors feature wavelength ranges for both transmission and reflection of light. A longpass dichroic mirror is highly reflective below the cutoff wavelength and highly transmissive above it, while a shortpass dichroic mirror is highly transmissive below the cutoff wavelength and highly reflective above it (35).

2.2.3 Multi-Photon Fluorescence Microscopy (MPFM)

Multi-photon excitation takes place via two- or three-photon absorption and was developed in its present form in 1990 by Denk (36), using high-power infrared lasers. But it was invented more than 60 years ago by Maria Goppert-Mayer in 1931 (37). Similar to CRM and CFM, the MPFM is a NIR laser scanning microscopy technique, but instead of recording the backscattered light, MPFM is based on nonlinear processes. Two-photon excitation fluorescence is the most commonly used nonlinear process, since the possibility of absorbing two photons is much higher than that of absorbing more than 2 photons (38).

Because two-photon excitation is a nonlinear process, where two photons have to be absorbed simultaneously (within $10^{-18}$ to $10^{-16}$ s), its probability of occurring is extremely low. A high laser power (high-photon density) is necessary. To obtain the high-photon densities necessary for the two-photon process, while keeping the average power sufficiently low in order not to harm the tissue, the excitation laser needs to be concentrated in both space and time. Therefore, a laser with short pulses, usually a femtosecond titanium-sapphire laser, is used for MPFM. Figure 2-3 is the illustration of the process of (a)
one-photon and (b) two-photon excitation.

Figure 2-3 Illustration of the process of (a) one-photon and (b) two-photon excitation

Figure 2-4 Layout of the conventional multi-photon fluorescence microscope (MPFM)
The layout of the conventional MPFM is similar with CFM except for the choice of the laser source as shown in Figure 2-4. The femtosecond laser which provides high photon density is needed for MPFM. The fluorescence detection can be more efficient because there is no need to exclude out-of-focus emission(38). The MPFM has the potential of becoming a noninvasive imaging technique and has been introduced for skin cancer diagnosis (39, 40).

2.2.4 Second Harmonic Generation (SHG)

Second Harmonic Generation (SHG) is also a nonlinear optical process, in which photons with the same frequency are "combined" to generate new photons with twice the energy of the initial photons. Similar to multi-photon excitation, higher-order processes (e.g. Third Harmonic Generation, Fourth Harmonic Generation, etc.) exist but are less common. The SHG was first demonstrated by Peter Franken in 1961(41).

![Figure 2-5 Illustration of the process of (a) Second-harmonic generation and (b) two-photon excitation](image-url)
The SHG and MPFM are based on different principles, despite sharing many features (as shown in Figure 2-5). SHG is based on excitation of electrons to an intermediate virtual state by absorbing a photon. This lifetime of this virtual state may last no longer than the quantum uncertainty, meaning the energy of the exciting photon influences how long the virtual state may exist. While at this excited virtual state, absorption of a second photon by the electron will result in further excitation to a higher-level real state. The electron will then decay back to its ground state, resulting in the emission of a photon with
doubled frequency. Two-photon excitation fluorescence involves excited real states rather than virtual states. The electron then decays to its ground state, resulting in the emission of a photon with a frequency less than double of its original, yet greater than the original one.

The layout of the conventional SHG is similar with MPFM except for adding a detector at the forward directions as shown in Figure 2-6. SHG signals are usually directional often only forward and backward depending on the material, whereas TPEF signals are isotropically emitted. Hence, two detectors are needed for SHG imaging.

2.3 Optical layout for KECK 3DFM 2.0

The KECK 3DFM underwent a major upgrade in the year 2013. The whole control system and part of the optical layout were redesigned. The performance of the second generation KECK 3DFM has been significantly improved after the upgrade.

The optical layout of 3DFM 2.0 is shown in Figure 2-7. The base of the microscope is a Nikon inverted TE2000U microscope. Two illumination sources are housed for imaging. A halogen lamp (orange path) is used for brightfield and DIC white light illumination, while a mercury lamp (gold path) is used for EFM. A maximum of six objective lenses can be mounted on the turret at one time.
Figure 2-7 Optical layout of KECK 3DFM 2.0
For EFM, specially designed filter cubes can be placed in the illumination path of the mercury lamp. Each cube contains the following: an excitation filter, to choose which spectral line in the mercury lamp will be used for illumination, a dichroic mirror, to reflect the excitation illumination and pass the emitted fluorescent light from the sample, and an emission filter, to block any light other than that from the fluorescent label desired. A turret houses a maximum of six filter cubes.

The sample holder is mounted on a piezoelectric crystal driven stage (PZ400, Piezosystems Jena), for controlling the sample in z-direction, and a translation stage (Proscan, Prior Scientific), for controlling the sample in x- and y-direction. The signals can be directed to various detectors according to the modality desired by using Switchable Mirror 2. A camera (Thorlabs DCC1545M) is used for acquiring EFM, DIC, and brightfield images. Another camera (RT900, SPOT) is used for detection of spectra.

Three lasers are used as illumination sources for all of the scanning modes. A mode-locked, femtosecond pulsed tunable titanium-sapphire laser (Tsunami, Spectra Physics) provides illumination in the near IR (700 - 1000 nm). The maximum average power is 1.5 watts when pumped with 8 watts of power from the pump laser (Millenia XsJ, Spectra Physics). The diameter of the output beam (red path) is 2 mm. Two Glan-Thompson polarizers (P1, P2 in Figure 2-7 are partially crossed to control the amount of illumination. The titanium-sapphire laser is served as the illumination source for the CRM, MPFM and SHG mode.
The argon laser (35-LAP-431-208, Melles Griot) and the Krypton-Argon laser (35-KAP-431-208) serve as the illumination sources for the CFM mode (purple path). The argon laser is “tunable" among discrete wavelengths between 454-514 nanometers. The argon laser produces a maximum of approximately 120 mW of power on the 488 nm and 514 nm spectral lines. The beam is horizontally polarized with a diameter of 0.7 mm. The Krypton-Argon laser is “tunable" among discrete wavelengths between 476 - 676 nanometers with maximum power 4 - 20 mW depending on the wavelength. The flip mirror controls the switch of the two lasers.

The dichroic, D1, combines both the titanium-sapphire and argon beams. Though the majority of the power reached the periscope after D1, a small portion (varies according to laser wavelength, but usually less than 10%) of the beams reached BS1, which is a beam splitter. The BS1 splits the beam into the Ocean Optics spectrometer (USB2000, Ocean Optics) for monitoring the laser spectrum, and into the power meter (Newport, 1830C) for real-time power monitoring. The majority of the laser beam passes through the BS2, a broadband polarizing beam splitter (PBS-450-1350-050, CVI), and reaches the polygon mirror scanner (SOS-SA24C, Lincoln Laser), which serves as the x-scanner. The galvanometer scanner serves as the y-scanner. The quarter wave plate is used for changing the polarization of input and output light to maximize the reflected light (dark red path) from the sample to reach the CRM APD (C5460, Hamamatsu) through the BS2.

The input laser beams are then directed into the Nikon microscope. The motorized rack (T-LLS, Zaber), controlled by a computer, delivers corresponding components according to the modalities used:
a mirror is delivered for the CFM mode, empty space is utilized for EFM, DIC, and brightfield modes, and a short pass dichroic is applied for MPFM and SHG modes. Note that the dichroic can also be used for EFM, DIC, and brightfield modes, as the dichroic passes visible light and reflects infrared light. The input beams then reach the sample through an objective mounted on the turret. Forward SHG signals (light blue path) are collected using a photomultiplier tube (PMT) (H9305, Hamamatsu). Another PMT (H9305-01, Hamamatsu) is used for detection of MPFM and backward SHG (light blue path).

The CRM signals (dark red path) reflected from the sample and reach the CRM APD through BS2, a polarizing beam splitter. Since the laser power needed in CFM, MPFM mode and SHG mode is much higher than that of CRM, to avoid saturation of the CRM APD, a polarizer, P3 is used to reduce the CRM signal reached to the APD. P4 is used to eliminate unwanted laser light reflected from the surface of the optical components.

The CFM signals (light green path) are collected by a third PMT (HC1024-02, Hamamatsu).

2.4 Control system for KECK 3DFM 2.0

2.4.1 Overview

The control system’s layout of the KECK 3DFM 2.0 is demonstrated in Figure 2-8. The 3DFM 2.0 consists of two computers: a 64 bit Windows 7 PC and a 32 bit Windows XP PC., one Image Acquisition board (IMAQ) card 1408, one NI USB-6341 Data Acquisition board (DAQ), several NI USB-6008 Data
Acquisition boards (DAQ) and a branch of optical parts as well as a few cameras.

The Windows XP PC is used due to incompatibility issues between the image acquisitions cards and the Windows 7 system. The Windows 7 computer controls the motorized rack through RS 232 port, and the grating (SP2150, Princeton Instruments), the NI USB-6341, Thorlabs camera as well as NI USB-6008 through USB.

The subsystem in the red rectangle is a 2-D scanning system which makes the laser to scan in two dimensions. The Polygon and galvanometric mirror scanner (Galvo) are responding for one dimension separately. The NI USB-6341 controls Polygon and Galvo to let them worked together by sending the HSync, VSync, and PCLK. IMAQ 1048 receives the picture signals and sync signals. Hsync, Vsync and Pclk are signals to control the acquisition processing (42, 43).
Figure 2-8 Layout of KECK 3DFM 2.0 control and electrical system
2.4.2 Laser scanning system

The block diagram of the laser scanning system of the KECK 3DFM 2.0 is demonstrated in Figure 2-9.

The NI USB-6341 is a data acquisition device as well as a programmable signal generator that contains 16 16-bit analog input channels, 2 16-bit analog output channels, 24 digital I/O lines, and 4 32-bit counter/timers for pulse-width modulation. The NI USB-6341 receives real-time input laser power signal from the power meter in analog form, and then converts and sends the sync signals to the Windows 7 PC. A 356.7 Hz square wave signal is generated by the NI USB-6341 for controlling of the polygon mirror scanner running at a speed of 200 RPS. The polygon mirror scanner contains 32 facets, and therefore multiplies the scanning speed to 6.4 kHz. The polygon mirror scanner sends a
Start-of-Scan (SOS) Signal at the beginning of each optical scan. For generation of the SOS signal, a red LED is placed close to the polygon mirror. The reflected red light is detected by two closely placed photodiodes. The SOS signal is the differential signal of the two photodiodes. The SOS signal is then processed by a bandpass filter, and turned into 6.4 kHz square waves, which is served as triggers for the NI USB-6341 to generate HSync, the signal that synchronizes each horizontal line of an image. The HSync also serves as the trigger for NI USB-6341 to generate VSync, the signal that synchronizes the acquisition of each image, pixel clock (PCLK), the signal that synchronizes the acquisition of each pixel, and the control signal that controls the galvanometric mirror scanner. Note that a pseudo-sawtooth is used in KECK 3DFM 2.0, which is different from the old KECK 3DFM. The control signal of the galvanometric mirror scanner will be discussed later.

The NI IMAQ PCI 1408 is a 4-channel image acquisition module that converts analog signals into 8-bit grayscale images. The NI IMAQ PCI-1408 is connected to the Windows XP PC through a PCI slot. The following detectors are connected to the four acquisition channels of NI IMAQ PCI-1408: CFM PMT, CRM APD, forward SHG PMT, and MPFM or backward SHG PMT. Since each NI IMAQ PCI-1408 contains only one A-D converter, only one channel can be running at a time. However, multiple modalities can be taken simultaneously by utilizing multiple NI IMAQ PCI-1408s. The number of channels is limited by the number of PCI slots in the computer, as well as the computer resources. An alternate solution is running different channels alternatively. e.g. we can acquire CRM and MPFM images alternatively. Since the image acquisition time is 0.1 s, the interval between the two modes is
0.1s, which results in a two-modal acquisition at a frame rate of 5 frame/s. The above solution is referred to as pseudo-simultaneous mode.

### 2.4.3 Power Supply Board

![Power Supply Board](image)

Figure 2-10 Power supply Board of KECK 3DFM control system

The power supply board provides the power supply to the Polygon, Galvo, APD, and the main control board. Figure 2-10 displays the photo of power supply board. The pins in the red rectangle area is designed for the old control board which is no longer used, but those pins are still functioning and can be used for others purposes if needed. The pins in the green rectangle area are designed for Galvo mirror control board. The blue rectangle covers the pins for powering the polygon while the yellow one is used for the APD of CRM mode.
2.4.4 Polygon Control Board

This board is the control board of polygon. A 356.7 Hz square wave signal is generated by the NI USB-6341 for controlling of the polygon mirror scanner running at a speed of 200 RPS. The polygon mirror scanner contains 32 facets, and therefore multiplies the scanning speed to 6.4 kHz. Figure 2-11 display the photo of polygon control board and Figure 2-12 demonstrates the function for each pin.

In practice, two pins are used for controlling the polygon. Pin1 is the reference frequency input which receives the square wave signal for controlling of the polygon speed. Pin 4 is the enable pin, which controls the ON (0) and OFF (1) state of the polygon.

![Figure 2-11 Polygon control board of KECK 3DFM control system](image)
2.4.5 Sensor and Filter

The main function of this part is obtaining the Start-of-Scan (SOS) signal from the Polygon. The SOS signal is generated at the beginning of each horizontal scan and is used to trigger the Hsync. The polygon contains 32 facets. Hence the frequency of the SOS signal is 32 times of the rotation speed of the polygon motor. The polygon mirror scanner sends a.

For generation of the SOS signal, a red LED is placed close to the polygon mirror. The reflected red light is detected by two closely placed photodiodes. The SOS signal is the differential signal of the two photodiodes. The SOS signal is then processed by a bandpass filter, and turned into 6.4 kHz square waves, which is served as triggers for the NI USB-6341 to generate HSync, the signal that synchronizes each horizontal line of an image.
The principle of this process is shown in Figure 2-13:

![Figure 2-13 The principle of the filter of KECK 3DFM control system](image)

In practice, an electrical bandpass filter is used for turning the SOS signal into square waves signal. Put output1 and output2 from the photodiode into CH1+ and CH1- of the filter. Use the Diff mode in CH1. (CH1: Low pass 150K Hz. Input Gain:50, Output Gain:0). Put the output of CH1 to CH2 input. (CH2: High pass 10 Hz. Input Gain:50, Output Gain:0). Put the CH2 into NI 6341 for triggering the Hsync. The input gain can change according to the different situation. Figure 2-14 demonstrate the single before (yellow curve) and after (blue curve) the filter.

![Figure 2-14 Signals before (yellow curve) and after (blue curve) filter](image)
2.5 The target system

2.5.1 Overview

Although the 3DFM 2.0 is a powerful tool providing various research functions, the polygon mirror scanner, which provides the scan in horizontal direction, does not provide the function of precise positioning. The complexity of the 3DFM 2.0 prevents further add-ons in the beam path. Therefore, a separate multi-modal microscope for targeting small particles (referred as the Target system) is built by Zhenhua Lai, and the author. The author is mainly responsible for the control system design. This device is mainly for studying of melanin and graphite.

The following modalities are included in the Target system: CRM, SMPAF, and brightfield. The SMPAF mode is similar to MPFM mode except that the illumination source must be a femtosecond pulsed laser in MPFM mode, while such requirement is not necessary in SMPAF mode.

2.5.2 Control system

The Target system is controlled by a 32-bit Windows 7 PC. The PC acquires brightfield images from the CCD through USB connection. The block diagram of the control system of the Target system is shown in Figure 2-15.

A NI USB-6251, connected through USB, is utilized to control the laser scanning modes. Similar to the NI USB-6341, the NI USB-6251 is a data acquisition device as well as a programmable signal
generator that contains 16 16-bit analog input channels, 2 16-bit analog output channels, 24 digital I/O lines, and 2 32-bit counter/timers for pulse-width modulation. The NI USB-6251 is programmed to generate HSync (square wave), PCLK (square wave), and the control signals (sawtooth wave) of both galvanometer mirror scanners. The control signal of the y-axis scanner also serves as V Sync through the analog trigger channel. The PC generates the CRM and SMPAF images according to the above signals as well as the signals, digitized by the NI USB-6251, from the APD and PMT. The PC is able to monitor the real-time power value of the power meter through the NI USB-6251.

Figure 2-15 Control system of the Target system

The galvanometer mirror scanners are designed for application of precise-positioning, rather than high-speed scanning, therefore, the frame rate of the laser scanning modalities is limited. To accommodate the image acquisition rate and the precision of the system, three modes are designed for
the laser scanning modalities:

a) Fast mode: the fast mode is designed for relatively higher speed but with poor linearity;

b) Slow mode: the slow mode is designed for good linearity but with acceptable speed;

c) Positioning mode: unlike the above two modes, the NI USB-6251 doesn’t generate HSync, PCLK, and the control signals in the positioning mode. Instead, the NI USB-6251 only generates DC signals corresponding to the location of each pixel. The pixel dwell time in this mode is 2 ms. Though with very slow speed, this mode provides the highest precision of the input laser position.

2.6 Conclusion

We upgraded the KECK 3DFM to 2.0 version. Part of the optical layout and the whole control system were redesigned. The programming language was changed from C# to LabVIEW. This change makes the image acquisition program more flexible and has the ability for users to extend the functionality. Since in LabVIEW, the subVIs can be easily assembled into VIs. Users can write their own control programs that are customized according to their own experiments.

Three new improvements have been achieved in 3DFM 2.0 besides the programming benefit: 1) the pixel resolution of the laser scanning modalities is increased from 640x480 to 800x600 which enlarge the field of view. 2) Multiple laser scanning modalities can be acquired simultaneously or pseudo-simultaneously, which significantly reduces the cost of time on multi-modal imaging and
enables *in vivo* multi-modal imaging. 3) The stability of the system has been increased. The frame rate of the system is more stable due to improved stability of control signals.

The Target system has been built specially for melanin SMPAF research. Three modalities are implemented in the Target system, including CRM, MPFM, and brightfield. The precise positioning function of the Target system is important to conducting the melanin SMPAF research.
3 Statistical studies of SMPAF of melanin

3.1 Introduction

The commonly known multi-photon fluorescence is usually a simultaneous excitation process, whereas the multi-photon activated fluorescence of melanin is a stepwise excitation process, which is similar to simultaneous excitation process, except that the intermediate states between the excited state and the ground state are real states.

The step-wise multi-photon activation fluorescence reveals a new method of melanin detection (14). Two benefits have been demonstrated in our previous studies (13, 14, 27, 28):

1) SMPAF requires an excitation energy two or more orders of magnitude lower than simultaneous excitation, while obtaining the same fluorescent level population density. Hence, The SMPAF can be generated using a low-cost continuous wave (CW) laser such as a diode laser. On the other hand, simultaneous two-photon excitation can only be generated using an expensive ultrafast pulsed laser.

2) SMPAF is a melanin-specific technique. There are several endogenous fluorophores in skin tissue; for example, NADH, keratin, melanin, collagen, elastin, which can all be excited by a femtosecond laser (38). Hence, it is difficult to distinguish each component from one another in an image acquired using a conventional multi-photon fluorescence microscope. In images of skin tissue acquired with SMPAF, only the melanin is excited with this unique fluorescence process, as far as we know.
In our early experiments, we have developed a model that describes the activation, excitation, and emission process of SMPAF as shown in Figure 3-1. When melanin is exposed to high power, it is changed into another form. This change occurs in the presence or absence of oxidizing agents, and can be accomplished with one photon of near-UV, two photons of mid-visible or 3 photons of near-IR radiation. Once the transition into this form has occurred, SMPAF of melanin can be induced by low power illumination. This form of melanin appears to be stable, and we can observed the melanin SMPAF signal after two days by using a low power (below the threshold of activation) laser.

Figure 3-1 (a) Activation and (b) Excitation models of SMPAF

Therefore, Activation is an essential processing for SMPAF. No melanin SMPAF can be obtained without the activation process. In this chapter, we examined the power dependence of the activation process of melanin SMPAF, as well as the photo-bleaching effect of melanin SMPAF. Also the regional analysis of melanin to study the spatial relationship in SMPAF was explored in this chapter.
3.2 Experiment materials and methods

3.2.1 Sample preparation

The eumelanin sample from Sepia (M2649-100MG) was purchased from the Sigma-Aldrich Corporation. Those samples contained the pure form of eumelanin, which is extracted from the ink sacs of the squid Sepia and has almost no other residues or components that could interfere by fluorescence. It served as the reference standard for our melanin sample. Individual granules of melanin could be observed and ranged in size from less than a micrometer to tens of micrometers and were mostly spherical or oval in shape. The sample comes in the form of a black powder and is insoluble in most solvents but could be spread on a microscope coverslip in a dry form.

To prepare for imaging, the samples were mounted inside culture dishes so that the air could be purged out with nitrogen to reduce photobleaching. 100% relative humidity on the sample was maintained by constantly running nitrogen bubbles through water. The eumelanin samples were suspended in a drop of deionized water and evaporated on a cover glass so that melanin particles were aggregated and were temporary not subjected to mechanical motion caused by periodic heating of the laser.

3.2.2 Experiment equipment

The Keck 3D fusion multimodal microscope 2.0 was used to image the samples in these experiments. A detailed description of the microscope system can be found in Chapter 2. CRM, MPFM
and brightfield modes are used and all of the imaging was done with Nikon Plan Apo microscope objective, 20 X 0.75 NA. The laser power was measured with a calibrated silicon photodiode (Newport, 1830-C) and all laser power levels refer to power measurements taken at the sample.

3.2.3 Experiment methods

Statistical studies results were obtained by modulating the laser activation power while detecting the fluorescence. The fluorescence image and laser level were recorded simultaneously and used to produce the activation power dependence plots. In these experiments, the sample was imaged with the polygonal-galvanometric laser scanning system and exposed with the same power laser within a certain time (60 seconds). The frame rate for fluorescence image detection is 10Hz, i.e. the image acquisition time for one single image is 0.1 second (800 x 600 pixels). In an effort to study morphological changes to the melanin particles due to the near-infrared radiation, the samples were also imaged in the brightfield and CRM modes.

3.3 Experiment results

3.3.1 SMPAF images

The selected melanin SMPAF images are shown in Figure 3-2; the power is \(8.9 \cdot 10^5 W/cm^2\) at sample.
Initially the laser was blocked by the shutter. After opening the shutter, the laser activated the melanin particles pixel by pixel lasting for 60 seconds. Figure 3-2(a-e) indicates the melanin SMPAF images of Frame1, Frame5, Frame 10, Frame 15, Frame 60, and Frame 250 correspondingly after
opening the shutter at the same location. Notice that the frame rate is 10Hz of KECK 3DFM microscope; one frame is for 0.1 second.

The melanin SMPAF signal can be observed from the first image frame after opening the shutter (Figure 3-2(a)). A small pixel number of melanin SMPAF were observed at this time. After 0.5 second opening the shutter, the pixel number and intensity of melanin SMPAF increased rapidly as shown in Figure 3-2(b). The pixel number and average intensity of melanin SMPAF reached the maximum peak at 0.5 second of the experiment. The melanin SMPAF number decreased they reach the peak as shown in Figure 3-2(c). The average size of melanin SMPAF region became smaller which revealed that photo-bleaching effect came about in this experiment. After 1.5 seconds, a large cluster of melanin SMPAF disappeared as shown with the rectangle in Figure 3-2(c) and the rectangle in Figure 3-2(d) which demonstrates that the melanin granules moved away or move to another layer in the experiment. After the 15ms, the number pixel of melanin SMPAF as well as the average size of the melanin SMPAF regions decreased slowly as shown in Figure 3-2(e) and Figure 3-2(f). The yellow circle in Figure 3-2(f) displays that the new melanin SMPAF can still be observed after turning off the shutter after 25 seconds. The average size of the melanin SMPAF regions did not vary much after 25 seconds until the end of the experiment.

3.3.2 Statistical studies of melanin SMPAF

The number of melanin SMPAF change versus time is shown in Figure 3-3; the laser intensity is
8.9 \times 10^5 \text{W/cm}^2 \text{ at sample. As mentioned in 3.3.1, the number of SMPAF arrived at the maximum peak quickly after opening the shutter (The 5th frame in this case). After 0.5 seconds, the melanin SMPAF number went down exponentially. Photo-bleaching occurred from the beginning of the experiment, spanning the entire duration until the end. After 40 seconds, the melanin SMPAF number remained quite stable, falling less than 10\% of the maximum florescence number.}

![Figure 3-3 Plots of the SMPAF pixel number versus time](image)

Selected power plots of melanin SMPAF number versus time are shown in Figure 3-4; the laser intensities are 11.8 \times 10^5 \text{W/cm}^2, 17.2 \times 10^5 \text{W/cm}^2, 23.5 \times 10^5 \text{W/cm}^2, and 30.2 \times 10^5 \text{W/cm}^2 \text{ at sample. The curves are normalized by dividing the maximum value of fluorescence number. After opening shutter, all the curves arrived at the maximum peak point in less than one second. Exponential decays are observed after reaching the maximum value in all the four curves due to the photo-bleaching effect. All the curves are decreased to 10\% of maximum number after 10 seconds except for the smallest power (11.8 \times 10^5 \text{W/cm}^2) (the blue curve).}
Figure 3-4 Selected normalized melanin SMPAF fractions *versus* time

Figure 3-5 Selected normalized decaying melanin SMPAF fractions *versus* time on ln scale

Figure 3-5 illustrates the selected power plots of decaying melanin SMPAF number versus time on ln scale, the laser intensities are $11.8 \times 10^5 W/cm^2$, $17.2 \times 10^5 W/cm^2$, $23.5 \times 10^5 W/cm^2$ and $30.2 \times 10^5 W/cm^2$ at sample. The plots suggest that varied decay rates can be observed with different laser
power. When the laser intensity increased, the decay rate increased simultaneously. After 60 seconds, small portions of melanin SMPAF remained. A relatively large portion of melanin is remained in the smaller power intensity since the smaller laser intensity has less photo-bleaching effect in the experiment. Intensive vibrations were observed in the gray curve in Figure 3-5 after 10 seconds due to the big movement of melanin particle at the scanning area which is contributed to high laser intensity.

Figure 3-6 displays the “climb time”, the time duration from opening the shutter to achieving the maximum value of the fluorescence signal for different powers. This time is a rough value due to the slow frame rate of the image acquisition card. For relatively small power, the rise time decreased quickly as the power increased since melanin particles may not be fully activated under such small power in a short time. While for the large power, the majority of melanin granules are activated in a short time.

![Figure 3-6 Times from opening the shutter to maximum peak point in different powers](image-url)
Figure 3-7 Times for decaying melanin fluorescence number to fall to one half of its initial value in different powers

Figure 3-7 shows the “half-life time” which means the times for decaying melanin fluorescence number to fall to one half of its initial value in different powers. The figure suggests that for the low power, the half-life time is longer than the high power. The half-life time didn’t change significantly if the power large than $12 \cdot 10^5 W/cm^2$ which indicates that the photo-bleaching rate did not change very much under higher laser intensity. Further discussion will be presented in Chapter 3.4.

3.3.3 Bright field and CRM

In an effort to study morphological changes to the melanin particles due to the near-infrared radiation, the samples were also imaged in the brightfield mode and CRM mode with high magnification and resolution.
Figure 3-8 (a) Brightfield image and (b) Confocal reflectance images of melanin in a same field of view (same as Figure 3-2)

Figure 3-8 (a) displays the brightfield image and (b) shows the CRM image; both images show the same field of view correspondingly. For brightfield mode in KECK 3DFM, the melanin granule blocked the light since the light source and camera are located at opposite sides. The melanin granule in this mode appear as black. While in CRM mode, the laser and detector are located at the same side of the
sample and light is reflected to the detector when they hit the melanin at the focal point. Therefore, the melanin granule appears as white in this mode.

Both the CRM and brightfield image can indicate the morphological and location information of melanin with different aspects of view. In CRM mode, only the layer at the focal plane is displayed in the image and specific layer structure can be obtained in this mode. While in brightfield mode, since all the layers are overlaid together, we are able to obtain the overview distributions and shapes of melanin particles in this mode.

In the statistical study, the number of melanin is acquired from both brightfield mode and CRM mode by selecting a threshold in the image. The melanin pixel value from brightfield mode is higher than CRM mode due to the factor that brightfield image contained the information with all the layers as mentioned in the last paragraph. Figure 3-9 plots the relative counting error between two modes in 27 independent regions. The relative counting error here is defined as \( e = \frac{N_{CRM} - N_{BRI}}{N_{BRI}} \), where \( N_{CRM} \) and \( N_{BRI} \) are the pixel numbers in the CRM and Brightfield images, respectively. The blue line in the figure shows the average error (11.9%) and all errors except one point (17.1%) are below 8% with the average error.
Figure 3-9 Relative error between CRM and Brightfield modes

Figure 3-10 Melanin SMPAF fractions with brightfield image (a) and CRM image (b)

Figure 3-10 (a) shows the combination results of the brightfield image and fluorescence records and (b) shows the combination results of the CRM image with fluorescence records. The y-axis for those two figures represents the normalized fractions of melanin fluorescence which is defined as the number of melanin SMPAF divided by the pixel number of melanin in the brightfield mode and CRM mode. The figures indicate that the proportion of activated melanin versus total melanin number. For example, Figure 3-10 (b) specifies that about 80% of melanin was activated under power $30.2 \cdot 10^5 W/cm^2$ less
than 1s. Notice that Figure 3-10 (a) and (b) have similar shape of curves within the same power, which demonstrates that the brightfield mode and CRM mode can provide similar melanin distribution information for analysis.

Figure 3-11 represents the max value of melanin SMPAF with the CRM mode image. The y-axis indicates the max portion of melanin being activated in different powers which is also the peak point value in Figure 3-10 (b). Further discussion will be presented in Chapter 3.4.

![Figure 3-11 Max value plot of melanin SMPAF fraction with CRM mode](image)

3.3.4 Region analysis

In this session, region analysis is taken in order to study the effect of size of melanin granule. Max
Intensity Projection (MIP) and Max Intensity Image (MII) were used for region segmentation as shown in Figure 3-12. The definition is demonstrated in the following paragraphs. Only the regions above 30 pixels are shown in the figure; the power is $8.9 \cdot 10^5 W/cm^2$ at sample with the same field of view as Figure 3-2.
Figure 3-12 (a) MIP segmentation result and (b) MII segmentation result

The Maximum Intensity Projection (MIP) consists of projecting the fluorescence signal volume with the highest value onto a 2D image. Because the MIP uses all of the fluorescence information throughout the entire experiment, it has two limitations. The first limitation is that it provides a large number of unpredictable regions, the majority of which is usually among the smallest in terms of physical size. Another limitation of the MIP is the greater amount of noise relative to the MII. Hence, MII is regarded as the standard for region segmentation.

For analyzing the Max Intensity Image (MII) image, first, calculate the number of melanin SMPAF versus time as shown in Figure 3-3 and then choose the time that has the maximum value of melanin pixel number (In this case, the fame number of the maximum value is 5). Second, do the max intensity projection in adjacent frames i.e. Frame 4, Frame 5 and Frame 6 of the fluorescence images in this case. Third, run the region analysis algorithm to decide each region. Typically, the average region size in MII is smaller than the region size in MIP since MIP contains large numbers of connections between the adjacent regions.
1622 regions are obtained from the MIP image while 918 regions are obtained from the MII image. Figure 3-13 presents the histogram of region size using MII. The ticks of the x-axis are customized for a better view since large portion of the regions enclose only a few pixels. Only the large regions which contain over 30 pixels are considered for analysis, since for the relatively small region, the granule moved quickly in the experiment which has a significant influence on the result.
The regions are classified into three types. Those types of regions’ fluorescence plots are shown on Figure 3-14. Figure 3-14(a) is called “burst” type. The melanin SMPAF appeared less than 0.5 second and then disappeared which shows the melanin granule moved away or moved to another layer. As the time went by, the melanin SMPAF in this region never appeared again. Figure 3-14(b) is called “natural” type. The fluorescence number versus time curve is similar to the curve on Figure 3-3. In this type, the melanin SMPAF signal reached the maximum value in a short time. Instead of disappearing, the
fluorescence signal decayed after the peak point. Figure 3-14(c) presents a “mix” type which is the combination of type1 and type2. In this type, the number of melanin SMPAF reached the peak and then disappeared. After a while (~3 to 5 seconds) the melanin fluorescence emerged again. The possible reason for this type is that after the melanin granule moved away to another layer, the melanin particle jumped back to the original position due to the laser thermal effect.

Among the 918 regions from the MII image, 187 of them have over 30 pixels (large region). Among those large regions, 121 of the regions are classified as “burst” type (64.7%), 46 (24.5%) followed “natural” type, and 20 (10.7%) demonstrated Type3 which is “mix” type.

For the “natural” type of melanin, the melanin particles remained static at their original locations. Only the photo-bleaching effect occurred for this type of melanin during the experiment. Therefore, the curve shapes of “natural” melanin are similar each other: after the pixel number of the melanin fluorescence reached their maximum value, they would decay exponentially due to the photo-bleaching effect. A curve fitting with an exponential function \( n = a \cdot e^{-bt} + c \) is used to determine the decay period of “natural” melanin, where \( n \) is the normalized pixel number of the melanin fluorescence in the region and \( t \) represents the time; \( a, b, c \) are three parameters in this exponential function. The physical meaning of these three parameters will be discussed in the following paragraphs.
(a) Power dependence curve of parameter “a”  

(b) Power dependence curve of parameter “b”  

(c) Power dependence curve of parameter “c”  

Figure 3-15  Power dependence curve of parameter a, b and c

Figure 3-15 indicates the three parameters in curve fitting function for the “natural” type of melanin. The figures show the mean value and the standard deviation of the coefficients with different power levels. Only the power level between $7.84 \cdot 10^5 \, W/cm^2$ to $12.32 \cdot 10^5 \, W/cm^2$ would be considered in the region used for the analysis experiment. At higher power levels, both the MII and the MIP in the fluorescence images have bad performance, for the region segmentation algorithms used.
Figure 3-15 (a) presents the parameter “a” in the curve fitting results. Since the normalized data points are selected as the original data, the “a” value should be approximately equal to 1. In practice, the start points in each data set are picked at the fifth frame time after the maximum value point time. The results show that the parameter “a” is not sensitive to the power level. Figure 3-15 (b) presents the parameter “b” in the curve fitting results. It represents the decay rate of the fluorescence number in the region. The mean value for “b” does not change very much based on power level, while the standard deviation is decreased when increasing the power. The results show a larger difference (standard deviation) in lower power levels than with relatively higher power levels. The plot shows that the activation of melanin is not uniform; the number of melanin in SMPAF may be affected by the structure and distribution of melanin granules, consistent with Figure 3-7. Figure 3-15 (c) presents the parameter “c” in the curve fitting. The parameter “c” demonstrates the remaining portion of melanin fluorescence after 60 seconds of exposure to the laser. Both the mean value and standard deviation of “c” become smaller when the laser power is increased, which indicates that an increase in laser power raises the risk of inducing the photo-bleaching effect.
Figure 3-16 Times from opening the shutter to maximum peak point in different powers among the region

Figure 3-16 shows the average “climb time”, the time duration from opening the shutter to achieving maximum value of the fluorescence signal, for different powers among the “natural” type of melanin’s region. The “climb time” decreased quickly as the power increased since melanin particles may not be fully activated under such small power in such a short period of time. This result is consistent with whole image results.

3.4 Analysis and discussion

Even though many studies have been done on the step-wise multi-photon fluorescence of melanin, the mechanism of this fluorescence is still poorly understood. The melanin goes through a step-wise multi-photon absorption process after the fluorescence has been activated with a high intensity laser.
No melanin step-wise multi-photon activation fluorescence can be obtained without the activation process. In this work, we have shown that melanin could be turned into an efficient emitter after first being activated with intense near-infrared light. Afterwards, the photo-bleaching effect occurred quickly after the fluorescence emission. The pixel number of melanin SMPAF reached its maximum peak after a few frames, regardless of power used, afterwards decaying due to the photo-bleaching effect. An increase in power or exposure time increased the amount of melanin activated, but increasing either parameter also raises the risk of photo-bleaching, in addition to added instability.

Different input powers have different decay rates as shown in Figure 3-4 and Figure 3-5. As the power increased, the decay rate simultaneously increased. However, the decay rate did not change significantly if the power was larger than $11 \cdot 10^5 W/cm^2$. One possible reason for this is the melanin SMPAF photo-bleaching effect is influenced by the density of melanin fluorescence. This fluorescence density did not change very much with the higher radiation intensity.

Figure 3-6 shows the time delay of melanin fluorescence from zero to the peak value. Generally, this takes between 100 milliseconds and 1 second varying depending on different power level. For conventional simultaneous multi-photon fluorescence, the fluorescence would reach the peak value in less than 100 nanoseconds, regardless of the power used (44). One possible reason for the longer delay in SMPAF compared to simultaneously multi-photon fluorescence is the activation process required in the SMPAF model. No SMPAF can be obtained without this multifaceted activation process, which is affected by both activation laser power and exposure time. As shown in Figure 3-6, for lower incident
laser power, the delay time is both related to the input laser intensity and the time. For higher incident laser power, the activation process is not affected as much by the input laser intensity, but rather by time.

The dashed red lines divide Figure 3-7 and Figure 3-11 into three regions. For smaller input power (power below $\sim 12 \cdot 10^5 W/cm^2$, region I), the melanin SMPAF intensity and amount increased gradually, while the half-life time quickly reduced. Since the activation of melanin is not uniform, the number of melanin SMPAF may be affected by the structure and distribution of melanin granules. When the laser focuses on the deeper layers, the photon would pass through the top layers and melanin granule on the top layer may scatter the light. Hence, a melanin particle at the edge would receive more energy than one in the center. For lower power, the melanin particles at the edges were activated, while the particles at the center did not receive enough power for activation. For intermediate power levels (power between $\sim 12 \cdot 10^5 W/cm^2$ to $\sim 22 \cdot 10^5 W/cm^2$, region II), the melanin SMPAF intensity and amount increased rapidly, while the half-life time slowly but surely shrank. At this power level, when the power went up, the number of melanin SMPAF increased linearly, while the rate of melanin photo-bleaching incrementally became smaller. Hence, the change of half-life time is smaller than it is for smaller power levels. For high power levels (power above $22 \cdot 10^5 W/cm^2$, region III), the melanin SMPAF intensity and amount increased gradually, since almost all the melanin particles are activated, while the half-life time stayed at a certain level. The melanin SMPAF behaves similarly to how it would if it were actually saturated.

We observed that the photo-bleaching effect in regional analysis started from the fringe of melanin
particles, moving towards the center. This was because the melanin particles at the edge would receive more laser energy than those in the center. Therefore, the center region of the target would have a longer lifetime compared to the outer regions. The photo-bleaching may be controlled by increasing the concentration of melanin particles.

Three different types of melanin fluorescence regions are defined on Figure 3-14. The differences come from the varied vibrational movement of particles within the melanin sample. For laser scanning system, the input laser beam hits the sample point by point. The thermal effects from absorption are what induce the melanin particles to jump about randomly. Therefore, the three types of melanin fluorescence regions represent three distinct kinds of particle movement. In the “burst” type, the melanin particles move away immediately after the exposure to the laser. While in the “natural” type, the melanin particles remained static. In the “mixed” type, the melanin particles move away after exposure to the laser, moving back to the original location. In the experiment, ~75% of the melanin particles behaved as either “burst” or “mixed” types, and ended up vibrating about, while the remaining 25% acted as the “natural” type, remaining more or less in the same location and layer.

The phenomenon of photo-bleaching, currently a very poorly understood phenomenon, occurs when a fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage and covalent modification (45). Our analysis study of melanin, the photo-bleaching effect occurred throughout the entire experiment. The photo-bleaching effect is affected by input the laser power, and may be thought of generally as broken up into three different performance regions, as discussed in
previous paragraphs in this section. In our experiment, the samples were mounted inside culture dishes so that the air could be purged with nitrogen to reduce photo-bleaching. Also, the photo-bleaching of SMPAF can be controlled by reducing the intensity or time-span of light exposure, simply by increasing the fluorophore concentration.

3.5 Conclusion

Melanin SMPAF activation and photo-bleaching effects are discussed in this chapter. We have shown that melanin could be turned into an efficient emitter after first being activated with intense near-infrared light. Afterwards, the photo-bleaching effect occurred quickly after the fluorescence emission. The pixel number of melanin SMPAF reached its maximum peak after a few frames, regardless of power used, afterwards decaying due to the photo-bleaching effect. There is a time delay of melanin fluorescence from zero to the peak value which is different with the conventional simultaneously multi-photon fluorescence. This time delay indicates that activation process is a different process with the excitation process which is consistent with our previous study (14). An increase in power or exposure time increased the amount of melanin activated, but increasing either parameter also raises the risk of photo-bleaching, in addition to added instability.

For power dependence of the activation processes, the input laser intensity is divided into three levels. Among those three levels, the photo-bleaching rate and melanin SMPAF fractions have different
relationships with the laser power. The photo-bleaching effect happened at from the beginning of the experiment. The number of melanin SMPAF may be affected by the structure and distribution of melanin granules. CRM and brightfield images are used in this experiment for studying morphological changes to the melanin particles. The results showed that both the CRM and brightfield images can indeed indicate the morphological information and spatial location of melanin particles with different aspects. By combining either CRM or brightfield with SMPAF, we can locate the melanin granules with respect to the other biological components. We have identified three distinct regions of melanin fluorescence by spatial regional study, each one differentiated by the movement of melanin particles within it.
4 Applications of SMPAF

4.1 *In vivo* SMPAF of melanin in human skin

4.1.1 Sample and equipment preparation

The images were collected from a 20-year old man with skin type IV. The imaged skin sites were from the forearms (Figure 4-1 (a)) and the thenar eminence (Figure 4-1(b)). A nevus is located in the thenar eminence. Both the health skin sites near the nevus and the nevus were imaged. An IRB exemption of this work has been issued by The Office of Human Subject Research Protection (HSRP) at Northeastern University.

![Figure 4-1](image)

Figure 4-1 The human skin imaged in the experiment

3DFM 2.0 is utilized for this work. The time interval between a CRM and a SMPAF image is 0.1 second. A water immersion 20 X 0.95 NA objective was used. The Ti:Sapphire Laser was tuned to CW mode at 830 nm to activate melanin, and to perform the SMPAF imaging. In this work the activation
laser intensity was \( \sim 4 \cdot 10^6 W/cm^2 \).

### 4.1.2 Experiment results

Figure 4-2 *In vivo* SMPAF images in human skin. Blue channel: CRM. Green channel:SMPAF. (a) Surface of the forearm; (b) The non-nevus part of the thenar eminence surface; (c) The nevus in the thenar eminence; (D) The nevus in the thenar eminence

All the images in Figure 4-2 are shown as composite of CRM (blue), which represents the physical structure of the skin, and SMPAF (green), which represents melanin. Figure 4-2 (a) is an image of the surface of the forearm shown in Figure 4-1(a). Figure 4-2 (b) is an image of the normal skin of thenar
eminence in Figure 4-1 (b). Figure 4-2 (c) and Figure 4-2 (d) are images of the nevus in the thenar eminence in Figure 4-1 (b). Figure 4-2 (c) and Figure 4-2 (d) contain significant higher numbers of green pixels than Figure 4-2 (a) and Figure 4-2 (b), which suggests that higher concentration of melanin in the nevus is being capture by the SMPAF imaging channel.

4.1.3 Discussion

This work demonstrates the feasibility of in vivo melanin detection using SMPAF in human skin (46). Further research needs to be done on the application of melanin SMPAF in clinical assessment of human skin. According to previous research, not all melanin in the field-of-view can be activated (14, 47). Therefore, it is very possible that the melanin observed in the SMPAF mode does not represent all melanin exist in the skin. The photophysics and photochemistry of activated states needs to be investigated to explain the nonuniformity of melanin activation.

4.2 Activation threshold of SMPAF

4.2.1 Introduction

In this experiment, we tested the activation threshold of various types of melanin samples to understand the activation processing of melanin SMPAF.

Activation is crucial in obtaining melanin SMPAF. No melanin SMPAF can be obtained without the activation process. A large photon density above the activation threshold is used to activate melanin
SMPAF. The activation threshold of melanin SMPAF, though varies by the type of melanin as well as the surrounding environment, is usually around $10^5 - 10^6 W/cm^2$. After activation, SMPAF signals can be detected below the activation threshold. Melanin SMPAF can be activated and excited equally well using pulsed and CW laser. The activation time varies among melanin particles, but is usually less than 60 s. The average activation time decreases when the laser power increases as we shown in Chapter 3.3. The activation of melanin doesn’t appear to be polarization dependent or wavelength dependent. However, the amount of melanin activated increases with incident power and exposure time, but increased exposure causes a risk of photo-bleaching and damage to the sample.

4.2.2 Experiment samples

Three kinds of melanin samples were used in this experiment.

1) Synthetic melanin in our lab (SIGMA M8631-100MG)

2) Fe-saturated melanin from Warren’s group of Duke University

3) EDTA washed melanin from Warren’s group of Duke University

The air samples were mounted inside microscope slides with cover glass. The N2 samples were mounted inside culture dishes (Delta T Bioptechs), with air purged overnight. We measured that both slices and dishes have similar transmission.
4.2.3 Experiment methods

We measured threshold of melanin by the following protocol:

1) Locate melanin using confocal reflectance microscopy (CRM) mode. The CRM and SMPAF are monitored simultaneously.

2) Increase the laser power gradually until a SMPAF signal is monitored.

3) Keep the laser exposure for ~1 min to make sure more SMPAF signals can be detected from other locations in the same field of view.

In this experiment, all tests were running using 920 nm pulsed laser with a 20x0.75 objective. Each sample was run on five distinct locations.
4.2.4 Experiment Results and analysis

Figure 4-3 Melanin SMPAF activation threshold in air and nitrogen

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th>Nitrogen</th>
<th>Difference between Air and N2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Threshold</td>
<td>Std</td>
<td>Threshold</td>
</tr>
<tr>
<td>Synthetic</td>
<td>3.39 (10^5 W/cm^2)</td>
<td>0.04</td>
<td>4.00 (10^5 W/cm^2)</td>
</tr>
<tr>
<td>Fe-saturated</td>
<td>4.11 (10^5 W/cm^2)</td>
<td>0.15</td>
<td>4.39 (10^5 W/cm^2)</td>
</tr>
<tr>
<td>EDTA washed</td>
<td>3.93 (10^5 W/cm^2)</td>
<td>0.17</td>
<td>4.41 (10^5 W/cm^2)</td>
</tr>
</tbody>
</table>

The experiment results are shown in Figure 4-3 and Table 4-1. Each point in the figure represents one test. Five tests were run on each sample.

We can see that the activation threshold varies according to the type of melanin as well as the
surrounding environment. The activation threshold undergoes ~10-20% increase when melanin is immersed in nitrogen compare to that in air, except the iron-saturated melanin. Simpson suggested that the activation of melanin SMPAF may be caused in part by the dissociation of metal ions or the selective degradation of iron-containing melanin (48). Therefore, the data may indicate that iron plays a role in the physical/chemical process of the activation of the melanin. The further experiment is needed to prove that such statement.

4.3 Conclusion

In this Chapter, we continue the research on the step-wise multi-photon activation fluorescence and explore the potential application for SMPAF.

The background-free feature of melanin detection using SMPAF has proved to be using in SMPAF image. The application of melanin SMPAF imaging for detection of melanin in human skin has been demonstrated. The acquired multi-modal images confirmed that the melanin SMPAF imaging is reliable. By combining CRM with SMPAF, we can locate the melanin with respect to other biological components. Considering the high performance and low-cost feature of the technology, melanin SMPAF is a promising technology for imaging melanin-related disease to assist diagnosis. The fact that SMPAF imaging has much higher signal-to-noise ratio under CW laser excitation as a result of reduced auto-fluorescence, while a CW laser is much cheaper than the pulsed laser, indicates that this new method can be relatively low cost.
In the experiment of activation threshold of SMPAF, we explore the various types of melanin samples’ SMPAF thresholds. The activation threshold varies according to the type of melanin as well as the surrounding environment. The results show that the activation threshold of synthetic melanin and EDTA washed melanin in N2 is greater than that in air, while the activation threshold of Fe-saturated melanin does not change when immersed in air and N2. The experiment results indicate that Fe is playing an important role in activation process of the SMPAF in different environments. Further experiment is needed to explore the mechanism of Fe in melanin SMPAF in detail.
5 Conclusions and future research

5.1 Conclusions

The author’s work can be divided into two aspects: 1) upgrade the KECK multi-modal microscope system and design the new target system and 2) the analysis study stepwise multi-photon activated fluorescence (SMPAF) of melanin.

Melanin is an important component of animal pigmentary systems, however the melanin has not been well understood, as it is an insoluble polymer without well-defined structure, which makes it difficult to isolate and study.

Multi-modal microscopy offers an alternative approach to study the melanin structure as well as melanin fluorescence with a branch of significant advantages. While each source of contrast provides unique advantages, a multimodal microscope will obtain the advantages of all.

The Keck 3D fusion multi-modal microscope (3DFM) in Optical Science Laboratory at Northeastern University allows us to image samples with multiple modalities on the same stage. The layout of KECK 3DFM can be customized according to the requirements of each project. We upgraded KECK 3DFM into second generation by redesigning the whole control system and part of the optical layout.

The performance of the second generation 3DFM has been significantly improved after the
upgraded from the following aspects:

1) The programming language for system control and image acquisition was changed from C# to LabVIEW. This change makes the image acquisition program more flexible and has the ability for users to extend the functionality.

2) The pixel resolution of the laser scanning modalities is increased from 640x480 to 800x600 which enlarge the field of view.

3) Multiple laser scanning modalities can be acquired simultaneously or pseudo-simultaneously, which significantly reduces the cost of time on multi-modal imaging and enables in vivo multi-modal imaging.

4) The stability of the system has been increased. The frame rate of the system is more stable due to improved stability of control signals.

5) Among the upgrade process, we use the NI USB-6341 which is commercial off-the-shelf instead of integrated circuit by specific design for controlling the laser scanning system. This change enlarged the system life cycle and made the system easy to maintenance.

The polygon mirror scanner in KECK 3DFM, which provides the scan in horizontal direction, does not provide the function of precise positioning. Hence, a novel precise positioning system (the target system) was built specially for melanin SMPAF research.
The objective for study the melanin fluorescence can be divided into two parts. One aspect of this project is a fundamental study of the underlying photophysics for fluorescence of melanin including the activation process, the photo-bleaching effect as well as the particle movement. The other aspect of this project is explore the design and optimization of a multi-modal microscope for detectability in human skin and other clinical imaging to label the different type of melanin.

The step-wise multi-photon activation fluorescence reveals a new method of melanin detection. The author continued this research for studying the activation process and photo-bleaching effect of melanin SMPAF. We explored activation process for melanin SMPAF. The melanin could be turned into an efficient emitter after first being activated with intense near-infrared light. Afterwards, the photo-bleaching effect occurred quickly after the fluorescence emission. The pixel number of melanin SMPAF reached its maximum peak after a few frames, regardless of power used, afterwards decaying due to the photo-bleaching effect. There is a time delay of melanin fluorescence from zero to the peak value which is different with the conventional simultaneously multi-photon fluorescence. This time delay indicates that activation process is a different process with the excitation process. An increase in power or exposure time increased the amount of melanin activated, but increasing either parameter also raises the risk of photo-bleaching, in addition to added instability. For power dependence of the activation processes, the input laser intensity is divided into three levels. Among those three levels, the photo-bleaching rate and melanin SMPAF fractions have different relationships with the laser power. The photo-bleaching effect happened at from the beginning of the experiment. The number of melanin
SMPAF may be affected by the structure and distribution of melanin granules. CRM and brightfield images are used in this experiment for studying morphological changes to the melanin particles. The results showed that both the CRM and brightfield images can indeed indicate the morphological information and spatial location of melanin particles with different aspects. We have identified three distinct regions of melanin fluorescence by spatial regional study, each one differentiated by the movement of melanin particles within it.

The application of melanin SMPAF imaging for detection of melanin inside human skin *in vivo* has been demonstrated. The acquired multi-modal images confirmed that the melanin SMPAF imaging is reliable. By combining CRM with SMPAF, we can locate the melanin with respect to other biological components. Considering the high performance and low-cost feature of the technology, melanin SMPAF is a promising technology for imaging melanin-related disease to assist diagnosis.

### 5.2 Future works

In the future, the research will continue in developing and improving the current technology of the multi-model microscopy. The operating for KECK 3DFM is still complicated due to its powerful functioning. Automation manipulation and easy to alignment is needed for future development. Also, the commercial components or instrument should be instead of the special design components for low-cost and easy-maintenance in the future.

Understanding the underlying mechanisms of step-wise multiphoton fluorescence is vitally
important for an understanding of melanin SMPAF. This in turn opens new doors to the discovery of potential applications for melanin related disease, first illuminating the cause(s), and with further work someday providing a remedy.

Electron Spin Resonance (ESR) spectroscopy would be a powerful tool to help us understand this activation process. For now, we defined three types of melanin SMPAF regions based on input intensity. Next, the melanin SMPAF activation process in several different target geometries is detailed, focusing primarily on the effect of region size. Specifically, SMPAF in smaller sized regions (pixel number < 30) is compared and contrasted.

Reflectance confocal microscopy offers an alternative approach with a significant advantage: directly showing nuclear and cellular detail, which are two key features examined by pathologists. We already show that by combining CRM with SMPAF, we can locate the melanin with respect to other biological components. This feature would greatly assist melanin detection, and additionally allow use of this technology for clinical imaging. One big challenge for combining CRM with SMPAF is that the majority of samples would move or jump due to the thermal effects from the laser during the detection process. This movement can be reduced by reducing the input power intensity. However, reducing the input power intensity increases the risk of failure for melanin activation. Therefore, a compromise is required to achieve the best of both worlds reduced movement and melanin activation will be a valuable project in the future.
Photo-bleaching in melanin is caused by SMPAF, and was present throughout the entire experiment. Removing this effect would make the results clearer. However, since this technology is able to selectively ablate melanin with high precision, leaving it alone might in fact be beneficial. In fact, it might assist us in building an application of melanin SMPAF for use in clinical assessment of human skin; this is a very important technique for cosmetic application.
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