MULTIMODAL MICROSCOPY AND
THE STEPWISE MULTI-PHOTON ACTIVATION
FLUORESCENCE OF MELANIN

A Dissertation Presented

By

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Abstract

The author’s work is divided into three aspects: multimodal microscopy, stepwise multi-photon activation fluorescence (SMPAF) of melanin, and customized-profile lenses (CPL) for on-axis laser scanners, which will be introduced respectively.

A multimodal microscope provides the ability to image samples with multiple modalities on the same stage, which incorporates the benefits of all modalities. The multimodal microscopes developed in this dissertation are the Keck 3D fusion multimodal microscope 2.0 (3DFM 2.0), upgraded from the old 3DFM with improved performance and flexibility, and the multimodal microscope for targeting small particles (the “Target” system). The control systems developed for both microscopes are low-cost and easy-to-build, with all components off-the-shelf. The control system have not only significantly decreased the complexity and size of the microscope, but also increased the pixel resolution and flexibility.

The SMPAF of melanin, activated by a continuous-wave (CW) mode near-infrared (NIR) laser, has potential applications for a low-cost and reliable method of detecting melanin.

The photophysics of melanin SMPAF has been studied by theoretical analysis of the excitation process and investigation of the spectra, activation threshold, and photon number absorption of melanin SMPAF.

SMPAF images of melanin in mouse hair and skin, mouse melanoma, and human black and white hairs are compared with images taken by conventional multi-photon fluorescence microscopy (MPFM) and confocal reflectance microscopy (CRM). SMPAF images significantly increase specificity and demonstrate the potential to increase sensitivity for melanin detection compared to MPFM images and CRM images.
Employing melanin SMPAF imaging to detect melanin inside human skin \textit{in vivo} has been demonstrated, which proves the effectiveness of melanin detection using SMPAF for medical purposes.

Selective melanin ablation with micrometer resolution has been presented using the Target system. Compared to the traditional selective photothermolysis, this method demonstrates higher precision, higher specificity and deeper penetration. Therefore, the SMPAF guided selective ablation of melanin is a promising tool of removing melanin for both medical and cosmetic purposes.

Three CPLs have been designed for low-cost linear-motion scanners, low-cost fast spinning scanners and high-precision fast spinning scanners. Each design has been tailored to the industrial manufacturing ability and market demands.
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1 Overview

The author’s work contains three major aspects: (1) multimodal microscopy, (2) the stepwise multi-photon activation fluorescence (SMPAF) of melanin, and (3) customized-profile lenses (CPL) for on-axis laser scanners.

The above topics are discussed in Chapters 2, 3, and 4, respectively.

Chapter 2 first introduces the microscopy modalities related to the author’s work, and then provides technical details on the Keck 3D fusion multimodal microscope 2.0 (3DFM 2.0) and the multimodal microscope for targeting small particles (the “Target” system).

Chapter 3 discusses the optical properties and the photophysics of melanin SMPAF, and presents the applications of melanin SMPAF in melanin detection and in selective laser ablation of melanin.

Chapter 4 depicts the principles, designs, manufacturing, and results of three CPLs for on-axis laser scanners. The three CPLs are designed for different applications.
2 Multimodal Microscopy

2.1 Introduction

Multimodal microscopy has become a popular topic due to the benefit of capturing images with multiple sources of contrast. While each source of contrast provides unique advantages, a multimodal microscope will obtain the advantages of all.

The Keck 3D fusion Multimodal microscope (3DFM) in the Optical Science Laboratory (OSL) at Northeastern University was first built by Daniel J. Townsend in 2004 (1). The 3DFM allows us to image samples with multiple modalities on the same stage (2). 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 layout of 3DFM can be customized according to the needs of each project. Multiple projects have been performed on 3DFM (3-6). Only the modalities related to the author’s projects will be introduced.

The 3DFM underwent a major upgrade in the year 2013. The upgrade to 3DFM 2.0 was led by the author and assisted by Zetong Gu, Stephen Karasek, and James Mclean. 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 Chapter 2.2.

Although the 3DFM 2.0 is a powerful tool providing various research functions, the polygon mirror scanner, which scans the laser beam in horizontal direction, does not provide the function
of positioning the beam at a specific location. It is difficult to integrate further add-ons to the beam path due to the complexity of the 3DFM 2.0. Therefore, a separate multimodal microscope for targeting small particles (referred as the “Target” system) was built by the author for studying of melanin, and is described in Chapter 2.3.

2.1.1 Epifluorescence microscopy (EFM)

![Diagram of an example epifluorescence microscope (EFM).](image)

Epifluorescence microscopy (EFM), usually with exogenous fluorophores, is commonly utilized for detecting the molecular signature of the samples. EFM is widely used in the life sciences because the cost is relatively low and the setup can be easily implemented into a conventional microscope.

The layout of an example EFM is shown in Figure 2-1. The excitation light is emitted from a strong white light source, and is filtered by an excitation filter. The dichroic beamsplitter reflects light with short wavelength (excitation light) and passes light with long wavelength
(fluorescence light). The objective lens is used as both the illumination condenser and the fluorescent light collector. The emission light is collected by the objective, filtered by the emission filter, and then recorded by a detector.

2.1.2 Confocal reflectance microscopy (CRM)

![Diagram of confocal reflectance microscopy](image)

Figure 2-2. Layout of a typical confocal reflectance microscope (CRM).

The confocal microscope was invented by Marvin Minsky in 1957 (7). By imaging with two similar lenses and placing an aperture (serving as a spatial filter) on the detector at a plane that is conjugate to the object plane, light scattered from out-of-focus planes is rejected by the spatial filter. The benefits of confocal microscopy include increased signal-to-noise ratio, the ability to collect serial optical sections from the sample, and quantitative measurements of the backscattered light from the specimen (8).
A confocal reflectance microscope (CRM) detects light that is backscattered into the objective lens. A simplified layout of a typical CRM is shown in Figure 2-2. The illumination light is collimated by the lens, reflected by the beamsplitter, and then focused to the sample through the objective. The backscattered light is collected by the same objective, passed through the beamsplitter, and then recorded by the detector. Out-of-focus lights (red and green) are rejected by the aperture on the detector.

If the light source is polarized, a quarter-wave plate can be placed between the objective and the beamsplitter; meanwhile, the beamsplitter should be a polarizing beamsplitter, which reflects light with one polarization and passes light with the 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 reaching the detector is maximized.

The signal reaching the detector only comes from the point of the sample that is conjugate to the aperture on the detector. Therefore, to create a 2D image, one can either move the sample or scan the input light in both horizontal and vertical directions.
2.1.3 Confocal fluorescence microscopy (CFM)


The layout of CFM is similar to that of CRM (Figure 2-3). Instead of using a polarizing beamsplitter in CRM, CFM uses a dichroic beamsplitter. The signal is further filtered by an emission filter. The out-of-focus fluorescence signal is rejected by the aperture on the detector.

2.1.4 Multi-photon fluorescence microscopy (MPFM)

Multi-photon excitation (the most common example is two-photon excitation) is a nonlinear optical process first predicted by Maria Goppert-Mayer in 1931 (9). Unlike the more commonly observed one-photon excitation fluorescence, which occurs when an electron in the ground state absorbs a higher energy photon to reach the excited state and then emits a lower energy photon, multi-photon excitation fluorescence occurs when an electron absorbs multiple lower energy
photons simultaneously to reach the excited state and then emits a higher energy photon. Note that in multi-photon excitation fluorescence, the sum of the energy of the input photons should be greater than that of the emission photon due to energy loss in the process. Since the probability of absorbing two photons simultaneously is much higher than that of absorbing more than 2 photons, two-photon excitation is much more common than higher-order processes. Therefore, most multi-photon excitation fluorescence is two-photon excitation fluorescence (TPEF). Figure 2-4 is a diagram explaining the physical principals of one-photon (Figure 2-4a) and two-photon (Figure 2-4b) excitation.

![Diagram of one-photon (a) and two-photon (b) excitation.](image)

Figure 2-4. Diagram of one-photon (a) and two-photon (b) excitation.

Since the probability of absorbing multiple photons simultaneously by an electron is very low, a large photon density is required to generate multi-photon fluorescence. Therefore, the generation of a multi-photon signal in most biological samples requires a femtosecond pulsed laser to meet the requirement of high peak photon density. The first multi-photon fluorescence microscope was developed in 1990 by Winfried Denk (10), eight years after the invention of femtosecond lasers.

The optical layout of MPFM is very similar to that of CFM, except that a femtosecond laser is used as the excitation source (Figure 2-5). Since MPFM signals only arise at the focal point with a small excitation volume, pinholes are not required in MPFM.
2.1.5 Second harmonic generation (SHG)

Second harmonic generation (SHG) was first demonstrated by Peter Franken et al. at the University of Michigan, Ann Arbor, in 1961 (11). It 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.

Although SHG shares many of the features of TPEF, SHG should not be confused with TPEF. Figure 2-6 shows the energy diagrams for both the SHG and TPEF processes. In SHG, an electron is excited to an intermediate virtual state by absorbing a photon. While at the excited virtual state, absorption of a second photon by the electron will result in further excitation to another virtual state at a higher level. The electron will then decay back to its ground state.
resulting in the emission of a photon with doubled frequency, whereas in TPEF, the excited state is a real state. The electron will decay to a lower state, which is also real, and then decay to its ground state resulting in the emission of a photon with a frequency less than double of its original but more than the original frequency. The lifetime of virtual states is \( \sim 10^{-16} \) s (12), whereas the lifetime of real states is usually a few nanoseconds (13).

![Energy diagrams for (a) second-harmonic generation (SHG) and (b) two-photon excited fluorescence (TPEF).](image)

Due to the difference in their physical mechanism, SHG signals and TPEF signals provide different features: (a) SHG signals have narrow emission bands which are always at half of the fundamental wavelength and tune with the input light, whereas the emission of TPEF is always longer than half of the fundamental wavelength and the emission band is usually stable and much wider. (b) SHG signals are usually directional (in most cases, only in forward- and backward-direction) depending on the material, whereas TPEF signals are usually isotropically emitted. (c) SHG signals are coherent, whereas TPEF signals are non-coherent.

The optical layout of SHG is very similar to that of MPFM, except that the signals from forward and backward directions can be both collected (Figure 2-7). Note that this setup can also be utilized for MPFM or EFM, but it is not necessary for MPFM and EFM to detect fluorescence signals on both directions.
2.2 The Keck 3D fusion multi-modal microscope 2.0 (3DFM 2.0)

2.2.1 Upgrade

Several minor upgrades have been performed since the 3DFM was built. More modalities have been added (14) and the control program has been revised many times, which enabled more functions on the 3DFM and created a powerful research tool. However, as the complexity of the system increased, all functions of the 3DFM were still integrated into a single control program, which was cumbersome; the stability and flexibility of the system was reduced. In addition, as the 3DFM had been used for a long time, the control system was old and replacement was
needed. Therefore, a major upgrade was important for the purpose of maintaining and improving the system.

In 3DFM 2.0, the optical layout was modified based on Townsend’s work. The control system had been redesigned. The language utilized to write the control program of the 3DFM 2.0 was changed from C# to LabVIEW for the following reasons: 1) the subVIs (equivalent to subprograms in C#) written in LabVIEW can be easily assembled into VIs (equivalent to programs in C#). 2) Instead of integrating all functions into a single program, subVIs of individual functions have been written. The subVIs can be assembled easily for different experiments. The stability of the system is increased by not including unnecessary functions into the control programs. 3) Compared to C#, LabVIEW is easier to learn for most engineers and scientists, therefore, users can write their own control programs that are customized according to their own experiments, which increased the flexibility of the system.

Besides the improvements in the control programs, the following improvements have been achieved in 3DFM 2.0: 1) the pixel resolution of the laser scanning modalities is increased from 640x480 to 800x600. 2) Multiple laser scanning modalities can be acquired simultaneously or pseudo-simultaneously, which significantly reduces the cost of time on multimodal imaging and enables in vivo multimodal 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.
2.2.2 Optical layout

Figure 2-8. The optical layout of 3DFM 2.0.
The optical layout of 3DFM 2.0 is shown in Figure 2-8. 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.

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 2-8) 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 reaches 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 beamsplitter. 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 beamsplitter (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 shortpass 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 near-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) are reflected from the sample and reach the CRM APD through BS2, a polarizing beamsplitter. 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).
2.2.3 Control system

Figure 2-9. The block diagram of the control system of 3DFM 2.0.
The block diagram of the control system of the 3DFM 2.0 is demonstrated in Figure 2-9. The 3DFM 2.0 is controlled by two computers: a 64 bit Windows 7 PC and a 32 bit Windows XP PC. The Windows XP PC is used due to incompatibility issues between the image acquisition cards and the Windows 7 system.

The Windows 7 computer controls the motorized rack through RS 232 port. The Windows 7 computer communicates with the following through USB connections: controls the grating (SP2150, Princeton Instruments) and the NI USB-6341, acquires EFM, DIC, and brightfield images from the Thorlabs camera, and receives real-time input laser power from the NI USB-6341. The grating creates a spectra image on the SPOT camera, which is connected to the image acquisition card that comes with the camera. The image acquisition card is connected to the Windows XP PC through a PCI slot. The Windows XP computer then processes the images into spectra data.

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 laser power signal input from the power meter in analog form, and then converts and sends the signals to the Windows 7 PC. A 356.7 Hz square wave signal is generated by the NI USB-6341 for controlling 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 a
6.4 kHz square wave signal, which serves as the trigger 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 3DFM 2.0, which is different from the old 3DFM. The control signal of the galvanometric mirror scanner will be discussed in Chapter 2.2.4.1.

The HSync, VSync, and PCLK are sent to the NI IMAQ PCI-1408, which 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 which 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 0.1 s, 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.

The gain of the MPFM or backward SHG PMT is controlled by the Windows XP PC through a RS 232 port. Besides the above PMT, the following components are also controlled through RS 232 ports: laser shutters, which are placed along the beam paths, the Jena
piezoelectric stage, and the Prior stage. The Ocean Optics spectrometer is connected to the Windows XP PC through USB connection.

### 2.2.4 Characterization of the laser scanning modalities

The characterization of the laser scanning modalities in 3DFM 2.0 is described in this section. The characterization of other modalities was performed by Townsend (1) and was not changed in 3DFM 2.0.

#### 2.2.4.1 Pixel resolution

A 10 Hz sawtooth wave was used for controlling of the galvanometric mirror scanner in the old 3DFM. As shown in Figure 2-10(a), after the galvanometric mirror scanner reached its positive maximum position, the scanner then retraced to its negative maximum position. An over-damping motion was created when the positive linear motion signal was applied to the scanner immediately after the negative retracing motion. Although easy to achieve electronically, this configuration created relatively long retracing time. The mechanical over-damping not only caused longer retracing time, but was also harmful to the scanner. Therefore, a relatively long retracing time is usually used if the scanner is controlled by sawtooth waves. There were 640 horizontal scans in each period of a vertical scan. However, 25% of the horizontal scans were discarded due to the over-damping of the galvanometric mirror scanner, which resulted in a pixel resolution of 640x480.

The retracing time can be significantly reduced by turning the over-damping motion of the galvanometric mirror scanner into critical damping (Figure 2-10(b)). The linear portion of the control signal in Figure 2-10(b) is similar to that of Figure 2-10(a). However, the portion that circled in red is changed, which is expanded in Figure 2-10(c). The scanner first retraces to its
negative maximum position, and then a parabola signal is applied to the scanner to create a constant force needed for critical-damping. Although an excess retracing signal is created between the retracing signal and the parabola signal, such signal is very short (0.16 ms), and therefore will not result in any additional motion. This configuration reduces the retracing time by 75%.

By applying the above scanner configuration as well as increasing the PCLK from ~5 MHz to 7.14 MHz, the pixel resolution is increased to 800x600.

Figure 2-10(d) and Figure 2-10(e) show two CRM images of a micrometer scale taken at the same location, using the signal shown in (a) and (b) respectively. The mechanical retracing time is much shorter in Figure 2-10(e). The retracing portions of the control signals in both cases are set as $5 \cdot T_h$ (0.78 ms), where $T_h$ is the time spent on each horizontal scan, which is 1/6400 s (~0.16 ms). The turning point in Figure 2-10(e) is much sharper than that in Figure 2-10(d), which indicates that critical damping occurs in Figure 2-10(e). Note that both images are uncropped. The retracing portions of all other presented images of the laser scanning modalities are cropped. A loud noise can be heard when operating the scanner using the signal in Figure 2-10(a) due to the short retracing time. Such noise can be avoided by either lengthening the retracing time or applying the signal shown in Figure 2-10(b).
Figure 2-10. Control signal of the galvanometric mirror scanner. (d) and (e) are uncropped CRM images taken at the same location, using the signal shown in (a) and (b) respectively. The mechanical retracing time is much shorter in (e).
2.2.4.2 Field of view (FOV)

The FOV is measured by imaging a stage micrometer scale (SM-3, Applied Image Inc.) with a 10x objective (Plan Fluor, Nikon) under CRM mode (Figure 2-11). The measured FOV is 554x415 um, with pixel size 0.692 um in both horizontal and vertical directions.

![Image of field of view](image)

Figure 2-11. The field of view (FOV) of the laser scanning modalities.

2.2.4.3 Laser power at the sample

The power measured by the power meter in Figure 2-8 needs to be converted to calculate the power that reaches the sample using the equation below:

\[ P_S = P_R \cdot C_f \cdot T_O \]

Equation 2-1

where \( P_S \) is the laser power at the sample, \( P_R \) is the power meter reading when placing the power meter at the location in Figure 2-8, \( C_f \) is the conversion factor, and \( T_O \) is the transmission of the
objective. The measurements of \( C_f \) and \( T_o \) are described in Chapter 2.2.4.3.1 and Chapter 2.2.4.3.2 respectively.

Note that the decay of input light due to the scattering and absorption of the sample is not considered in this work.

### 2.2.4.3.1 Conversion factor

The conversion factors, \( C_f \), are measured by placing the power meter before the objective and then dividing the values when placing the power meter at the location in Figure 2-8. The results are shown in Table 2-1. Results are measured with the quarter-wave plate in and out of the beam path.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Conversion Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Quarter-wave Plate</td>
</tr>
<tr>
<td>800</td>
<td>4.62</td>
</tr>
<tr>
<td>830</td>
<td>18.05</td>
</tr>
<tr>
<td>880</td>
<td>9.88</td>
</tr>
<tr>
<td>920</td>
<td>10.45</td>
</tr>
</tbody>
</table>

### 2.2.4.3.2 Transmission of objectives

The transmission of the objectives cannot be measured directly by the power meter due to the short working distance of the objectives. Therefore, an optical layout, described in Figure 2-12, is created for measuring the transmission of the objectives. The input laser light is reflected by the dichroic used for MPFM mode and then reaches a mirror, which is placed on the sample holder. The reflected light passes through the objective and the MPFM dichroic, and then is measured by the power meter. The power meter reads the value with and without the objective.
for calculation of the objective transmission. The equation for calculating the objective transmission is derived below:

The value read by the power meter without the objective is:

\[ P_{wo} = P_{in} \cdot R_d \cdot R_m \cdot T_d, \]

Equation 2-2

where \( P_{in} \) is the input laser power, \( R_d \) is the reflection coefficient of the dichroic, \( R_m \) is the reflection coefficient of the mirror, \( T_d \) is the transmission of the dichroic.

The value read by the power meter with the objective is:

\[ P_o = P_{in} \cdot R_d \cdot R_m \cdot T_d \cdot T_o^2, \]

Equation 2-3

where \( T_o \) is the transmission of the objective.

Dividing Equation 2-2 by Equation 2-3, we have:

\[ T_o^2 = \frac{P_o}{P_{wo}}, \]

Equation 2-4

Therefore,

\[ T_o = \sqrt{\frac{P_o}{P_{wo}}}. \]

Equation 2-5
Figure 2-12. The optical layout for measuring the transmission of objectives.

The transmission of the objectives is shown in Table 2-2.

Table 2-2. The transmission of the objectives.

<table>
<thead>
<tr>
<th>Objective</th>
<th>Transmission (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>830 nm</td>
</tr>
<tr>
<td>10x 0.30 NA plan Fluor</td>
<td>73.85</td>
</tr>
<tr>
<td>20x 0.75 NA plan Apo</td>
<td>87.37</td>
</tr>
<tr>
<td>40x 0.65 NA Plan</td>
<td>59.23</td>
</tr>
<tr>
<td>40x 0.80W NA Fluor</td>
<td>56.64</td>
</tr>
</tbody>
</table>

2.3 The multimodal microscope for targeting small particles (the Target System)

Although the 3DFM 2.0 is a powerful tool providing various research functions, the polygon mirror scanner, which scans in the 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 multimodal microscope for targeting small particles (referred as the Target system) is built by the author for studying of melanin.

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. The details of the SMPAF signal of melanin and graphite will be discussed later.
2.3.1 Optical layout

Figure 2-13. Optical layout of the multimodal microscope for targeting small particles (the Target system).
The optical layout of the Target system is described in Figure 2-13. A 975 nm continuous wave (CW) laser (LU0975M500, Lumics) (red path), controlled by a diode controller (LDC 3742, ILX Lightwave), serves as the illumination source of the CRM (dark red path) and SMPAF (light green path) mode. The laser beam is first expanded and collimated by a beam expander and then passes a 9:1 beamsplitter. A small portion of the beam is delivered to the power meter for real-time power monitoring. The 900 nm shortpass dichroic is intended for future experiments, which is not included in this work. The first galvanometer scanner (z1913, General Scanning Inc.) scans the beam in x-axis. The Telescope 1 is used as a relay lens. The mirror, M4, turns the beam to vertical direction for y-scan. The second galvanometer scanner (z1913, General Scanning Inc.) scans the beam in y-axis, and therefore, a 2D scan is created. The Telescope 2 is used as a beam expander as well as a relay lens. The switchable mirror is utilized for switching between the brightfield mode and the laser scanning modes. The sample is mounted on translational stages for moving in x-, y-, and z-direction and for tiling the sample in θ-, φ-, and ψ-directions.

The SMPAF and CRM signals are collected by the objective (Plan Fluor, Nikon), and delivered to the SMPAF PMT (R928, Hamamatsu), through an 800 nm longpass dichroic, and to the CRM APD (C5460, Hamamatsu), through the 9:1 beamsplitter. The size of the pinhole used for the CRM mode is 40 um.

A halogen lamp serves as the illumination source of the brightfield mode (orange path). The brightfield images are collected by a CCD camera (DCC1545M, Thorlabs). Since the images reached the CCD are reversed images, the images are reversed again by software.
2.3.2 Control system

As shown in Figure 2-14. The Target system is controlled by a 32-bit Windows 7 PC. The PC acquires brightfield images from the CCD through USB connection.

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 VSync through the analog trigger channel. The PC generates the CRM and SMPAF images according to the above signals and 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.

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 relatively poor linearity;

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

c) **Positioning mode**: unlike the above two modes, the NI USB-6251 doesn’t generate H SYNC, 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.

Though the scanning speeds of the three modes are different, they target the same field of view with different linearity. The specifications of the three modes are compared in Table 2-3.

<table>
<thead>
<tr>
<th>Mode</th>
<th>x-scanner speed (Hz)</th>
<th>PCLK (Hz)</th>
<th>Image acquisition time (s)</th>
<th>Linearity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast</td>
<td>121</td>
<td>69696</td>
<td>3</td>
<td>Poor</td>
</tr>
<tr>
<td>Slow</td>
<td>10</td>
<td>3850</td>
<td>36.3</td>
<td>Good</td>
</tr>
<tr>
<td>Positioning</td>
<td>1.43</td>
<td>500</td>
<td>245</td>
<td>Good</td>
</tr>
</tbody>
</table>
2.3.3 Characterization

The FOV of the Target system is measured by the same micrometer scale mentioned previously. The measured FOV is shown in Table 2-4.

Table 2-4. Field of view measurements for the Target system.

<table>
<thead>
<tr>
<th>Modality</th>
<th>FOV (um)</th>
<th>Pixel Resolution</th>
<th>Pixel Size (um)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightfield</td>
<td>1800 x 1440</td>
<td>1280 x 1024</td>
<td>1.41</td>
</tr>
<tr>
<td>CRM and SMPAF</td>
<td>350 x 350</td>
<td>350 x 350</td>
<td>1</td>
</tr>
</tbody>
</table>

The power conversion factor of the Target system is shown in Figure 2-15. The power conversion factor is measured by placing the power meter after the objective.

![Figure 2-15](image_url)
A linear fit of the data shown in Figure 2-15 is performed using Origin. The fitted curve is represented by the equation:

\[ P = 0.08857 \cdot I - 5.33453 \]

where \( P \) is the power at the sample with unit in mW, and \( I \) is the corresponding controller current with unit in mA.

### 2.4 Conclusion

3DFM has been upgraded to 3DFM 2.0. Part of the optical layout and the whole control system has been redesigned. The flexibility and stability of 3DFM 2.0 has increased compared to 3DFM. The pixel resolution has been increased from 640x480 in 3DFM to 800x600 in 3DFM 2.0. Simultaneous and pseudo-simultaneous multimodal imaging features have been added to 3DFM 2.0, which enabled \textit{in vivo} multimodal imaging of biological samples.

The Target system has been built specially for melanin SMPAF research. Three modalities are implemented in the Target system, including CRM, SMPAF, and brightfield.

The control systems developed for both 3DFM 2.0 and the Target system are low-cost and easy-to-build, with all components off-the-shelf. The system has not only significantly decreased the complexity and the size of the microscope, but also increased the pixel resolution and flexibility of the microscope.

The development of both systems has fostered biomedical research, especially the study of melanin SMPAF, which will be demonstrated in Chapter 3.
3 The Stepwise Multi-Photon Activation Fluorescence of Melanin

3.1 Introduction

3.1.1 Melanin

Melanin, an ubiquitous biological pigment produced by melanocytes in most organisms (15, 16), is an important component of animal pigmentary systems (17-19). Melanin has not been well-understood, as it is an insoluble polymer without well-defined structure, which makes it difficult to isolate and study (20).

Naturally occurring pigmentation that determines hair, eye and skin coloration is attributed to two types of melanin: eumelanin and pheomelanin. Eumelanin is the dominant component of brown and black pigments in dark skin and black hair, while pheomelanin is more common in yellow and red pigments in hair (21). Eumelanin is the most prevalent and important form of melanin, and has been the most intensively studied (19, 22). All melanin samples used in this work are eumelanin unless stated specifically.

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 (23); melasma, an acquired brown hypermelanosis of the face (24); solar lentigines and ephelides, benign pigmented spots which are associated with an increased risk of skin cancer (25-27); 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 (28, 29).

X-ray scattering and tunneling microscopy experiments have suggested that oligomers of four to six indole species, termed “protomolecule”, form and may represent a fundamental
structural unit of eumelanin (30, 31). Theoretical models of eumelanin protomolecules and their optical properties have also been proposed (32). Atomic force and scanning electron microscopy have shown that the organization of melanin into various sizes, from tens of nanometer to micrometers, plays an important role in its functions and photophysics (33). Optical spectroscopy is a commonly used method of studying the optical properties of melanin (16, 34). Near-infrared autofluorescence imaging shows that cutaneous melanin in pigmented skin disorders emits higher NIR autofluorescence than surrounding normal tissue (35).

### 3.1.2 Detection of melanin in microscopy level

Various microscopy methods have been used to study melanin. CRM is well-known for “optical sectioning” of tissue, which is the imaging of thin sections of the sample at both high resolution and high contrast without physically dissecting the tissue (36-38). CRM has been used for detection of melanin, providing strong contrast (36). However, the specificity and sensitivity of melanin detection using CRM is low due to the background signal from other high-contrast materials. Other methods of microscopic melanin detection require the use of pulsed lasers. e.g. MPFM lifetime imaging (39), pump-probe imaging (40, 41).

Previous research has shown that step-wise multi-photon excitation fluorescence (SMPEF) of melanin can be generated by 120 fs-pulse NIR laser with an emission spectrum that peaks at around 525 nm (42, 43). The process of stepwise two-photon excitation of melanin fluorescence is different from the generally known process of simultaneous two-photon excitation of fluorescence. The former is a two-step process via a real intermediate excitation state, while the latter lacks the real intermediate state. Stepwise two-photon excitation requires excitation intensity two or more orders of magnitude lower than simultaneous excitation to obtain the same population density of the fluorescence (5, 43-45). Therefore, simultaneous two-photon excitation
can only be generated by an ultrafast laser, which is very expensive. In contrast, stepwise two-photon excitation can be generated using a continuous wave (CW) laser, which is usually low-cost. e.g. a diode laser (5, 45). Details of the differences between simultaneous excitation and stepwise excitation will be discussed later in this chapter.

3.1.3 The stepwise multi-photon activated fluorescence (SMPAF) of melanin

The melanin SMPEF observed by Teuchner and Hoffmann was weak and difficult to detect in hair and skin due to the background interference from other biological components (5). Kerimo et al. discovered enhanced melanin fluorescence by stepwise three-photon excitation (44). The author has defined this process as stepwise multi-photon activated fluorescence (SMPAF) to distinguish the process discovered by Kerimo from the SMPEF observed by Teuchner and Hoffmann, which does not require the activation step (5). SMPAF can be activated using a low-cost CW laser with a SMPAF signal much stronger than SMPEF and simultaneous multi-photon excitation of fluorescence. The melanin SMPAF spectra showed a broad distribution from 450 nm to 1000 nm and beyond with a peak at 960nm (5, 45). The number of photons absorbed by melanin SMPAF varies depending on the activation laser power (46).

An important advantage of SMPAF is the ability to acquire background-free detection of melanin. Mega first used melanin SMPAF combined with CRM in a low-cost, CW imaging configuration (47). Lai first utilized melanin SMPAF to obtain melanin images in mouse skin and hair (5), mouse melanoma (48), human hair, and human skin in vivo (49). Lai also first applied melanin SMPAF to guide selective melanin ablation for biomedical and cosmetic purposes, which will be discussed in this dissertation.
3.1.4 Overview

The difference between the stepwise multi-photon excitation and the commonly known simultaneous multi-photon excitation is explained in Chapter 3.2. The lifetime of the intermediate states and the excited state of activated melanin is calculated and estimated using rate equations in Chapter 3.2.

The samples used in this work are introduced in Chapter 3.3.

The activation process is crucial in generating the melanin SMPAF signal. The activation process is studied and discussed in Chapter 3.4. 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 (50). Experiments have been performed to prove that such statement is partially true, which can be found in Chapter 3.4.1. The measurements of melanin EPR signals before and after activation indicates that free radicals have been removed from melanin during the activation process, which is presented in Chapter 3.4.2.

The SMPAF is unique in melanin compared to other biological components in skin. Therefore, melanin SMPAF demonstrates very high specificity in melanin detection. The specificity of melanin SMPAF is shown in Chapter 3.5.

The melanin SMPAF has a broad spectrum extending from the visible spectra to the NIR. The spectrum of various melanin samples are demonstrated in Chapter 3.6.

Kerimo has shown that the photon number absorbed in melanin SMPAF is 3 when activated using low laser power \((10^5 \sim 10^6 \text{ W/cm}^2)\). However, a mixture of 3-order process and 2-order process exist in mouse hair, which is demonstrated in Chapter 3.7. The photon number
absorption of melanin SMPAF when activated using high laser power \(2 \cdot 10^6 \text{ W/cm}^2\) is also demonstrated in Chapter 3.7.

As mentioned previously in this chapter, an important advantage of SMPAF is the ability to acquire background-free detection of melanin. The application of melanin SMPAF imaging for detection of melanin in mouse hair and skin, mouse melanoma, and human skin has been demonstrated in Chapter 3.8. Selective activation of melanin in sepia skin is also demonstrated in Chapter 3.8.

The activation of melanin SMPAF requires high laser power. The damage to samples caused by long-time high-power laser exposure is studied in Chapter 3.9. Though the unwanted laser damage should be avoided, selective laser ablation of melanin has numerous applications in treatment of melanin-related skin diseases and in cosmetics. SMPAF guided selective ablation of melanin is capable of achieving micrometer resolution and is relatively low-cost. The SMPAF guided selective ablation of melanin is performed in sepia skin and is shown in Chapter 3.9.

### 3.2 Theoretical background

#### 3.2.1 Stepwise excitation vs. simultaneous excitation

As mentioned previously in this chapter, 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 the simultaneous excitation process, except that all the intermediate states between the excited state and the ground state are real states. Figure 3-1 shows an example of the comparison between the simultaneous three-photon excitation and the stepwise three-photon excitation. In simultaneous excitation, lifetime of the
virtual states is estimated to be $\sim 10^{-16}$ s (51). The lifetime of the real states are much longer (usually $\sim 10^{-9}$ s).

The energy diagrams shown in Figure 3-1 follow the rate equations below:

$$\frac{d}{dt} N_1 = R_{01} \cdot I(t) \cdot (N_0 - N_1) - R_{12} \cdot I(t) \cdot (N_1 - N_2) - \frac{N_1}{T_1},$$

Equation 3-1

$$\frac{d}{dt} N_2 = R_{12} \cdot I(t) \cdot (N_1 - N_2) - R_{23} \cdot I(t) \cdot (N_2 - N_3) - \frac{N_2}{T_2},$$

Equation 3-2

$$\frac{d}{dt} N_3 = R_{23} \cdot I(t) \cdot (N_2 - N_3) - R_3 \cdot I(t) \cdot N_3 - \frac{N_3}{T_3},$$

Equation 3-3

where $N_0$, $N_1$, $N_2$, and $N_3$ are the electron population density of State 0 (ground state), 1, 2, and 3 (excited state) respectively. Here, we suppose $N_0$ is constant. $R_{01}$, $R_{12}$, and $R_{23}$ are the transition
rates from State 0 to State 1, State 1 to State 2, and State 2 to State 3 respectively. \( R_3 \) is the decay rate of the excited state. \( T_1, T_2, \) and \( T_3 \) are the lifetime of State 1, 2, and 3 respectively. \( I(t) \) is the intensity of the input laser.

To populate electrons from State 0 to State 3, we need to have:

\[
N_0 \gg N_1 \gg N_2 \gg N_3,
\]

Equation 3-4

Therefore, Equation 3-1 - Equation 3-3 can be simplified as:

\[
\frac{d}{dt} N_1 = R_{01} \cdot I(t) \cdot N_0 - \frac{N_1}{T_1},
\]

Equation 3-5

\[
\frac{d}{dt} N_2 = R_{12} \cdot I(t) \cdot N_1 - \frac{N_2}{T_2},
\]

Equation 3-6

\[
\frac{d}{dt} N_3 = R_{23} \cdot I(t) \cdot N_2.
\]

Equation 3-7

a) Pulsed laser

\[
I(t)
\]

\[
0 \quad \tau \quad T_L \quad T_L + \tau \quad t
\]

Figure 3-2. Profile of a pulsed laser. \( \tau \): pulse width; \( T_L \) period.
Suppose the input laser is a pulsed laser, with pulse width \( \tau \), period \( T_L \), and intensity \( I(t) \) as shown in Figure 3-2.

i) **Simultaneous excitation**

The pulse width of the Tsunami laser is \( \sim 10^{-13} \) s, which is much longer than the lifetime of virtual states, but shorter than that of the excited states (usually \( \sim 10^{-9} \) s). Then we have

\[
T_1, T_2 \ll \tau \ll T_3.
\]

Equation 3-8

At \( t \in (0, \tau) \), \( I(t) \gg 0 \), from Equation 3-5, we have:

\[
N_1 = C_1 \cdot e^{-t/T_1} + R_{01} \cdot N_0 \cdot e^{-t/T_1} \cdot \int e^{t/T_1} \cdot I(t) \cdot dt,
\]

Equation 3-9

where \( C_1 \) is a constant.

Since

\[
\int e^{t/T_1} \cdot I(t) \cdot dt = T_1 \cdot e^{t/T_1} \cdot I(t) - T_1 \cdot \int e^{t/T_1} \cdot d[I(t)],
\]

Equation 3-10

we have

\[
N_1 = C_1 \cdot e^{-t/T_1} + R_{01} \cdot N_0 \cdot T_1 \cdot I(t) - R_{01} \cdot N_0 \cdot T_1 \cdot e^{-t/T_1} \cdot \int e^{t/T_1} \cdot d[I(t)] \approx R_{01} \cdot N_0 \cdot T_1 \cdot I(t).
\]

Equation 3-11

Here, as from Equation 3-8, \( T_1 \ll \tau \), hence \( C_1 \cdot e^{-t/T_1} \approx 0 \).
To prove that

\[ R_{01} \cdot N_0 \cdot T_1 \cdot I(t) \gg R_{01} \cdot N_0 \cdot T_1 \cdot e^{-\frac{t}{T_1}} \cdot \int e^{\frac{t}{T_1}} \, d[I(t)], \]

Equation 3-12

we need to prove

\[ I(t) \gg e^{-\frac{t}{T_1}} \cdot \int e^{\frac{t}{T_1}} \, d[I(t)], \]

Equation 3-13

which is to prove

\[ I(t) \cdot e^{\frac{t}{T_1}} \gg \int e^{\frac{t}{T_1}} \cdot \frac{d[I(t)]}{dt} \cdot dt. \]

Equation 3-14

Since

\[ I(t) \cdot e^{\frac{t}{T_1}} = \int \frac{d\left[I(t) \cdot e^{\frac{t}{T_1}}\right]}{dt} \cdot dt = \int e^{\frac{t}{T_1}} \cdot \frac{d[I(t)]}{dt} \cdot dt + \frac{1}{T_1} \cdot \int e^{\frac{t}{T_1}} \cdot I(t) \cdot dt, \]

Equation 3-15

to prove Equation 3-14, we only need to prove

\[ \frac{1}{T_1} \cdot \int e^{\frac{t}{T_1}} \cdot I(t) \cdot dt \gg 0. \]

Equation 3-16

Equation 3-16 is true since \( \frac{1}{T_1} \), \( e^{\frac{t}{T_1}} \), and \( I(t) \) are all \( \gg 0 \).

We rewrite Equation 3-11 below as:
\[ N_1 \approx R_{01} \cdot N_0 \cdot T_1 \cdot I(t). \]

Equation 3-17

Similarly, from Equation 3-6 and Equation 3-17, we have

\[
N_2 = C_2 \cdot e^{-\frac{t}{\tau_2}} + R_{12} \cdot e^{-\frac{t}{\tau_2}} \cdot \int e^{\frac{t}{\tau_2}} \cdot I(t) \cdot N_1 \cdot dt
\]

\[
\approx C_2 \cdot e^{-\frac{t}{\tau_2}} + R_{12} \cdot R_{01} \cdot N_0 \cdot T_1 \cdot e^{-\frac{t}{\tau_2}} \cdot \int e^{\frac{t}{\tau_2}} \cdot [I(t)]^2 \cdot dt
\]

\[
\approx R_{12} \cdot R_{01} \cdot N_0 \cdot T_1 \cdot T_2 \cdot I(t)^2.
\]

Equation 3-18

where \( C_2 \) is a constant.

From Equation 3-7 and Equation 3-18, we have

\[
N_3 = R_{23} \cdot \int I(t) \cdot N_2 \cdot dt \approx R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot T_1 \cdot T_2 \cdot \int I(t)^3 \cdot dt.
\]

Equation 3-19

At \( t \in (\tau, T_L) \), \( I(t) = 0 \), though there is no input photons, State 3 is populated. Emission will occur following the equation below:

\[
\frac{d}{dt} N_3 = -\frac{N_3}{T_3}.
\]

Equation 3-20

Therefore

\[
N_3 = -\int_{\tau}^{T_L} \frac{N_3}{T_3} \, dt = N_3^0 \cdot e^{-\frac{t-\tau}{T_3}},
\]

Equation 3-21
where $N_3^0$ is the electron population density of State 3 at time $\tau$, which is

$$
N_3^0 = R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot T_1 \cdot T_2 \cdot \int_0^\tau I(t)^3 \, dt = R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot T_1 \cdot T_2 \cdot \langle I(t)^3 \rangle \cdot T_L.
$$

Equation 3-22

Therefore, the detected fluorescence signal is

$$
F \propto \langle N_3(t) \rangle = \frac{1}{T_L} \int_0^{T_L} N_3 \, dt = \frac{1}{T_L} \int_0^{T_L} N_3^0 \cdot e^{-\frac{t-\tau}{T_3}} \, dt = \frac{N_3^0}{T_L} \cdot T_3 \cdot \left(1 - e^{-\frac{T_L-T_3}{T_3}}\right) \approx \frac{N_3^0}{T_L} \cdot T_3.
$$

Equation 3-23

By combining Equation 3-22 and Equation 3-23, we have

$$
F \propto \langle N_3(t) \rangle \approx R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot T_1 \cdot T_2 \cdot T_3 \cdot \langle I(t)^3 \rangle,
$$

Equation 3-24

which means

$$
F \propto \langle I^3 \rangle.
$$

Equation 3-25

ii) Stepwise excitation

In stepwise excitation, the laser pulse width is much shorter than the lifetime of the intermediate states (usually $\sim 10^{-9}$s). Then Equation 3-8 is changed to

$$
\tau \ll T_1, T_2, T_3.
$$

Equation 3-26

At $t \in (0, \tau), I(t) \gg 0$, Equation 3-1- Equation 3-3 can be simplified as:
\[
\frac{d}{dt}N_1 = R_{01} \cdot I(t) \cdot N_0,
\]
Equation 3-27

\[
\frac{d}{dt}N_2 = R_{12} \cdot I(t) \cdot N_1,
\]
Equation 3-28

\[
\frac{d}{dt}N_3 = R_{23} \cdot I(t) \cdot N_2.
\]
Equation 3-29

From Equation 3-27

\[
N_1 = R_{01} \cdot N_0 \cdot \int I(t) \, dt.
\]
Equation 3-30

From Equation 3-28 and Equation 3-30,

\[
N_2 = R_{12} \cdot I(t) \cdot R_{01} \cdot N_0 \cdot \int I(t) \cdot \left[ \int I(t) \, dt \right] \, dt = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int I(t) \, dt \right]^2.
\]
Equation 3-31

From Equation 3-29 and Equation 3-31,

\[
N_3 = R_{23} \cdot I(t) \cdot \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \int I(t) \cdot \left[ \int I(t) \, dt \right]^2 \, dt
= \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int I(t) \, dt \right]^3.
\]
Equation 3-32

At \( t \in (\tau, T_L) \), \( I(t) = 0 \), Equation 3-1 - Equation 3-3 can be simplified as:
\[
\frac{d}{dt} N_1 = -\frac{N_1}{T_1},
\]
Equation 3-33

\[
\frac{d}{dt} N_2 = -\frac{N_2}{T_2},
\]
Equation 3-34

\[
\frac{d}{dt} N_3 = -\frac{N_3}{T_3}.
\]
Equation 3-35

From Equation 3-33,

\[
N_1 = -\int_\tau^{T_L} \frac{N_1}{T_1} \, dt = N_1^0 \cdot e^{-\frac{t-\tau}{T_1}},
\]
Equation 3-36

where \(N_1^0\) is the electron population density of State 1 at time \(\tau\), which is

\[
N_1^0 = R_{01} \cdot N_0 \int_0^\tau I(t) \, dt = R_{01} \cdot N_0 \cdot \langle I(t) \rangle \cdot T_L.
\]
Equation 3-37

Similarly, from Equation 3-34 and Equation 3-37,

\[
N_2 = N_2^0 \cdot e^{-\frac{t-\tau}{T_2}},
\]
Equation 3-38

where
\[ N_2^0 = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \left[ \int_0^\tau I(t) \, dt \right]^2 = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \langle I(t) \rangle^2 \cdot T_L^2. \]

Equation 3-39

From Equation 3-35 and Equation 3-39,

\[ N_3 = N_3^0 \cdot e^{-\frac{t-\tau}{T_3}}, \]

Equation 3-40

where

\[ N_3^0 = \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \left[ \int_0^\tau I(t) \, dt \right]^3 = \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \langle I(t) \rangle^3 \cdot T_L^3. \]

Equation 3-41

Therefore, the detected fluorescence signal

\[ F \propto \langle N_3(t) \rangle = \frac{1}{T_L} \int_0^{T_L} N_3 \, dt = \frac{1}{T_L} \int_0^{T_L} N_3^0 \cdot e^{-\frac{t-\tau}{T_3}} \, dt = \frac{N_3^0}{T_L} \cdot T_3 \cdot \left( 1 - e^{-\frac{T_L-\tau}{T_3}} \right) \]

\[ \approx \frac{N_3^0}{T_L} \cdot T_3 \cdot \left( 1 - e^{-\frac{T_L}{T_3}} \right), \]

Equation 3-42

which means

\[ F \propto N_3 \propto \langle I \rangle^3. \]

Equation 3-43

b) CW laser
Assuming that the system has reached equilibrium under the exposure of CW laser with intensity $I$, we have

$$\frac{d}{dt}N_1 = \frac{d}{dt}N_2 = \frac{d}{dt}N_3 = 0.$$  

Equation 3-44

Therefore, Equation 3-5 - Equation 3-7 can be simplified as:

$$N_1 = R_{01} \cdot I \cdot N_0 \cdot T_1,$$

Equation 3-45

$$N_2 = R_{12} \cdot I \cdot N_1 \cdot T_2 = R_{12} \cdot R_{01} \cdot I^2 \cdot N_0 \cdot T_2 \cdot T_1,$$

Equation 3-46

$$N_3 = R_{23} \cdot I \cdot N_2 \cdot T_3 = R_{23} \cdot R_{12} \cdot R_{01} \cdot I^3 \cdot N_0 \cdot T_3 \cdot T_2 \cdot T_1,$$

Equation 3-47

which means

$$F \propto N_3 \propto I^3.$$  

Equation 3-48

Equation 3-47 is also consistent with Equation 3-24.

c) Conclusion

Although Equation 3-25 and Equation 3-43 appear similar to each other, the difference is significant under a femtosecond pulsed laser. Take the Tsunami laser as an example, with pulsed width $\sim 10^{-13}$s and period 12.5 ns,
\[ \frac{\langle I^3 \rangle}{\langle I \rangle^3} \sim 10^{10}. \]

Equation 3-49

Therefore, stepwise excitation can be easily achieved using a CW laser, whereas simultaneous excitation requires a pulsed laser.

### 3.2.2 Intermediate states of melanin SMPAF

Use Equation 3-47 divided by Equation 3-42, we have

\[
\frac{N_{3\text{CW}}}{N_{3\text{pulsed}}} = \frac{R_{23} \cdot R_{12} \cdot R_{01} \cdot I^3 \cdot N_0 \cdot T_3 \cdot T_2 \cdot T_1}{\frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot \langle I(t) \rangle^3 \cdot T_L^2 \cdot T_3 \cdot \left(1 - e^{-\frac{T_L}{T_3}}\right)} = \frac{6 \cdot T_2 \cdot T_1}{\left(1 - e^{-\frac{T_L}{T_3}}\right) \cdot T_L^2}.
\]

Equation 3-50

According to Kerimo’s previous report (44), the signal strength of melanin SMPAF signal is the same order when using CW or pulsed mode of the Tsunami laser. For the Tsunami laser, \( T_L \approx 10^{-8}s \), suppose \( T_1 \approx T_2 \), then we have

\[ T_1 \approx T_2 \approx 10^{-9}s. \]

Equation 3-51

which is consistent with our previous estimate.

To further understand the lifetime of the intermediate states of melanin SMPAF, we designed an optical delay as shown in Figure 3-3(a). The laser beam coming from the pulsed laser (red path) is separated into two branches by BS1. The yellow path went through a longer path compared to that of the blue path, so that a delay of \( \Delta t \) is created between the two paths. The tunable neutral density filter is able to control the intensity of the yellow path. The yellow and blue paths are then merged by BS2 to create a new beam.
The profile of the new beam is shown in Figure 3-3(b). Two pulses are created at each period $T_L$.

![Diagram of experimental setup](image)

Figure 3-3. Studying of the intermediate states of melanin SMPAF by an optical delay line. (a) Experimental setup; (b) Profile of the output laser beam.

At $t \in (0, \tau), I(t) = I_1(t) \gg 0$, from Equation 3-30 - Equation 3-32,

$$N_1 = R_{01} \cdot N_0 \cdot \int I_1(t) \, dt,$$

Equation 3-52

$$N_2 = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int I_1(t) \, dt \right]^2,$$

Equation 3-53
\[ N_3 = \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int I_1(t) \, dt \right]^3. \]

Equation 3-54

At \( t \in (\tau, \tau + \Delta t) \), \( I(t) = 0 \), from Equation 3-36 - Equation 3-41

\[ N_1 = N_1^0 \cdot e^{-\frac{t-\tau}{\tau_1}}, \]

Equation 3-55

where

\[ N_1^0 = R_{01} \cdot N_0 \int_0^\tau I_1(t) \, dt; \]

Equation 3-56

\[ N_2 = N_2^0 \cdot e^{-\frac{t-\tau}{\tau_2}}, \]

Equation 3-57

where

\[ N_2^0 = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2; \]

Equation 3-58

\[ N_3 = N_3^0 \cdot e^{-\frac{t-\tau}{\tau_3}}, \]

Equation 3-59

where
\[ N_3^0 = \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^3. \]  

Equation 3-60

When \( t \in (\tau + \Delta t, 2\tau + \Delta t) \), \( I(t) = I_2(t) \). Use

\[ t' = t - (\tau + \Delta t). \]  

Equation 3-61

Then \( t' \in (0, \tau) \). From Equation 3-5, we have:

\[ N_1 = C_1 \cdot e^{-t' \frac{\Delta t}{\tau_1}} + R_{01} \cdot N_0 \cdot e^{-t' \frac{\Delta t}{\tau_1}} \int e^{\frac{t'}{\tau_1}} \cdot I_2(t') \cdot dt'. \]  

Equation 3-62

where \( C_1 \) is a constant.

For \( t' = 0 \), which is \( t = \tau + \Delta t \), from Equation 3-55, Equation 3-56 and Equation 3-62, we have

\[ N_1(t' = 0) = N_1^{01} = N_1^0 \cdot e^{-\frac{\Delta t}{\tau_1}} = e^{-\frac{\Delta t}{\tau_1}} \cdot R_{01} \cdot N_0 \int_0^\tau I_1(t) \, dt = C_1. \]  

Equation 3-63

Therefore

\[ N_1 = R_{01} \cdot N_0 \int_0^\tau l_1(t) \, dt \cdot e^{-\frac{\Delta t + t'}{\tau_1}} + R_{01} \cdot N_0 \cdot e^{-\frac{t'}{\tau_1}} \cdot \int e^{\frac{t'}{\tau_1}} \cdot I_2(t') \cdot dt'. \]  

Equation 3-64

Since \( t' < \tau \ll T_1 \), we have
\[ N_1 \approx R_{01} \cdot N_0 \cdot e^{-\frac{\Delta t}{T_1}} \int_0^\tau I_1(t) \, dt + R_{01} \cdot N_0 \cdot \int I_2(t') \, dt'. \]

Equation 3-65

Similarly, from Equation 3-6, we have:

\[ N_2 = C_2 \cdot e^{-\frac{t'}{T_2}} + R_{12} \cdot e^{-\frac{t'}{T_2}} \cdot \int e^{\frac{t'}{T_2}} \cdot I_2(t') \cdot N_1 \cdot dt'. \]

Equation 3-66

For \( t' = 0 \), from Equation 3-57, Equation 3-58 and Equation 3-66,

\[ N_2(t' = 0) = N_2^{01} = N_2^0 \cdot e^{-\frac{\Delta t}{T_2}} = e^{-\frac{\Delta t}{T_1}} \cdot N_2^0 = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2 = C_2. \]

Equation 3-67

Therefore, from Equation 3-65, Equation 3-66 and Equation 3-67

\[ N_2 = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t + t'}{T_2}} + R_{12} \cdot e^{-\frac{t'}{T_2}} \cdot \int e^{\frac{t'}{T_2}} \cdot I_2(t') \cdot \left[ R_{01} \cdot N_0 \cdot e^{-\frac{\Delta t}{T_1}} + R_{01} \cdot N_0 \cdot \int I_2(t') \, dt' \right] \, dt'
\]

\[ = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t + t'}{T_2}} + R_{12} \cdot R_{01} \cdot N_0 \cdot e^{-\frac{t'}{T_2}} \cdot \int e^{\frac{t'}{T_2}} \cdot \left[ \int_0^\tau I_1(t) \, dt \right] \cdot I_2(t') \, dt' + R_{12} \cdot R_{01} \cdot N_0 \cdot e^{-\frac{t'}{T_2}} \cdot \int e^{\frac{t'}{T_2}} \cdot I_2(t') \, dt' \]

\[ \cdot \int e^{\frac{t'}{T_2}} \cdot \left[ \int I_2(t') \, dt' \right] \cdot I_2(t') \, dt'. \]

Equation 3-68

Since \( t' < \tau \ll T_1, T_2 \), we have
\[ N_2 \approx \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t}{\tau_2}} + R_{12} \cdot R_{01} \cdot N_0 \cdot e^{-\frac{\Delta t}{\tau_1}} \cdot \left[ \int_0^\tau I_1(t) \, dt \right] \cdot \int I_2(t') \, dt' + R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int I_2(t') \, dt' \right] \cdot I_2(t') \cdot dt' \]

\[ = \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t}{\tau_2}} + R_{12} \cdot R_{01} \cdot N_0 \cdot e^{-\frac{\Delta t}{\tau_1}} \cdot \left[ \int_0^\tau I_1(t) \, dt \right] \cdot \int I_2(t') \, dt' + \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int I_2(t') \, dt' \right]^2. \]

Equation 3-69

From Equation 3-7, we have:

\[ N_3 = C_3 \cdot e^{-\frac{t'}{\tau_5}} + R_{23} \cdot e^{-\frac{t'}{\tau_3}} \cdot \int e^{\frac{t'}{\tau_5}} \cdot I_2(t') \cdot N_2 \cdot dt'. \]

Equation 3-70

For \( t' = 0 \), from Equation 3-59, Equation 3-60 and Equation 3-70, we have

\[ N_3(t' = 0) = N_3^{01} = N_3^0 \cdot e^{-\frac{\Delta t}{\tau_5}} = e^{-\frac{\Delta t}{\tau_5}} \cdot \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^3 = C_3. \]

Equation 3-71

Therefore,
\[ N_3 = e^{-\frac{\Delta t}{T_3}} \cdot \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left( \int_0^\tau l_1(t) \, dt \right)^3 \cdot e^{-\frac{t'}{T_3}} + R_{23} \cdot e^{-\frac{t'}{T_3}} \]

\[ \int e^{\frac{t'}{T_3}} \cdot l_2(t') \]

\[ \left\{ \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t}{T_2}} + R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right] \]

\[ \int l_2(t') \cdot dt' + \frac{1}{2} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int l_2(t') \, dt' \right]^2 \cdot dt' \]

\[ = \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} \cdot e^{-\frac{t'}{T_3}} + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \]

\[ \left[ \int_0^\tau l_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t}{T_2}} \cdot e^{-\frac{t'}{T_3}} \cdot \int e^{\frac{t'}{T_3}} \cdot l_2(t') \cdot dt' + R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \]

\[ \int_0^\tau l_1(t) \, dt \cdot e^{-\frac{\Delta t}{T_1}} \cdot e^{-\frac{t'}{T_3}} \cdot \int e^{\frac{t'}{T_3}} \cdot l_2(t') \cdot \int l_2(t') \, dt' \cdot dt' + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \]

\[ \cdot N_0 \cdot e^{-\frac{t'}{T_3}} \cdot \int e^{\frac{t'}{T_3}} \cdot l_2(t') \cdot \left[ \int l_2(t') \, dt' \right]^2 \cdot dt'. \]

Equation 3-72

Since \( t' < \tau \ll T_1, T_2 \), we have
\[
N_3 \approx \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2 \\
\cdot e^{-\frac{\Delta t}{T_2}} \cdot \int I_2(t') \, dt' + R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \int_0^\tau I_1(t) \, dt \cdot e^{-\frac{\Delta t}{T_1}} \\
\cdot \int I_2(t') \cdot \left[ \int I_2(t') \, dt' \right]^2 \, dt' \\
= \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \\
\cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t}{T_2}} \cdot \int I_2(t') \, dt' + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \int_0^\tau I_1(t) \, dt \cdot e^{-\frac{\Delta t}{T_1}} \\
\cdot \left[ \int I_2(t') \, dt' \right]^2 + \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int I_2(t') \, dt' \right]^3 \cdot \\
\]

Equation 3-73

When \( t \in (2\tau + \Delta t, T_L) \), \( I(t) = 0 \). From Equation 3-7

\[
N_3 = N_3^{02} \cdot e^{-\frac{t'-\tau}{T_3}},
\]

Equation 3-74

where
\[ N_3^{02} = \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^3 e^{-\frac{\Delta t}{T_3}} + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_1(t) \, dt \right]^2 \]
\[ \cdot e^{-\frac{\Delta t}{T_2}} \cdot \int_0^\tau I_2(t') \cdot dt' + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \int_0^\tau I_1(t) \, dt \cdot e^{-\frac{\Delta t}{T_1}} \]
\[ \cdot \left[ \int_0^\tau I_2(t') \cdot dt' \right]^2 + \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau I_2(t') \cdot dt' \right]^3. \]

Therefore, the detected fluorescence signal

\[ F \propto \langle N_3(t) \rangle = \frac{1}{T_L} \cdot \int_0^{T_L} N_3 \, dt = \frac{1}{T_L} \cdot \left( \int_0^{\Delta t+\tau} N_3^0 \cdot e^{-\frac{t-\tau}{T_3}} \, dt + \int_\tau^{T_L-\Delta t-\tau} N_3^{02} \cdot e^{-\frac{t-\tau}{T_3}} \, dt \right) \]
\[ = \frac{T_3}{T_L} \cdot \left[ N_3^0 \cdot \left( 1 - e^{-\frac{\Delta t}{T_3}} \right) + N_3^{02} \cdot \left( 1 - e^{-\frac{T_L-\Delta t-2\tau}{T_3}} \right) \right]. \]

Equation 3-76

Again \( t' < \tau \ll T_1, T_2 \), then

\[ \langle N_3(t) \rangle \approx \frac{T_3}{T_L} \cdot \left[ N_3^0 - N_3^0 \cdot e^{-\frac{\Delta t}{T_3}} + N_3^{02} - N_3^{02} \cdot e^{-\frac{T_L-\Delta t}{T_3}} \right]. \]

Equation 3-77

From Equation 3-60 and Equation 3-75,
\[ \langle N_3(t) \rangle \approx \frac{T_3}{T_L} \left\{ \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 - \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 \right\} \cdot e^{-\frac{\Delta t}{T_3}} + \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} \]

\[ \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t}{T_2}} \cdot \left[ \int_0^\tau l_2(t') \, dt' \right] + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} \]

\[ \cdot \left[ \int_0^\tau l_2(t') \, dt' \right]^2 + \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} + \frac{1}{2} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^3 \cdot e^{-\frac{\Delta t}{T_3}} \]

\[ \cdot \left[ \int_0^\tau l_2(t') \, dt' \right]^2 + \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot \left[ \int_0^\tau l_2(t') \, dt' \right]^3 \right\} \cdot e^{-\frac{\Delta t}{T_3}} \}

\[ = \frac{R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot T_3}{T_L} \]

\[ \cdot \left\{ \frac{1}{6} \left[ \int_0^\tau l_1(t) \, dt \right]^3 + \frac{1}{2} \left[ \int_0^\tau l_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t}{T_2}} \cdot \left[ \int_0^\tau l_2(t') \, dt' \right] + \frac{1}{2} \cdot \left[ \int_0^\tau l_1(t) \, dt \right]^2 \cdot e^{-\frac{\Delta t}{T_2}} \cdot \left[ \int_0^\tau l_2(t') \, dt' \right] + \frac{1}{2} \cdot e^{-\frac{\Delta t}{T_2}} \cdot \left[ \int_0^\tau l_2(t') \, dt' \right]^3 \right\} \cdot \frac{T_{T_3}}{T_{T_3}} \}

By definition

Equation 3-78
\[ \langle l_1(t) \rangle = \frac{1}{T_L} \cdot \int_0^T l_1(t) \, dt, \]

Equation 3-79

\[ \langle l_2(t) \rangle = \frac{1}{T_L} \cdot \int_0^T l_2(t') \, dt'. \]

Equation 3-80

From Equation 3-78, Equation 3-79 and Equation 3-80, we have

\[ \langle N_3(t) \rangle = \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot T_3 \cdot T_L^2 \]

\[ \cdot \left[ (l_1(t))^3 \cdot \left( 1 - e^{-\frac{T_L}{T_3}} \right) + 3 \cdot (l_1(t))^2 \cdot (l_2(t)) \cdot e^{-\frac{\Delta t}{T_3}} \cdot \left( 1 - e^{-\frac{T_L - \Delta t}{T_3}} \right) + 3 \right. \]

\[ \left. \cdot (l_1(t)) \cdot (l_2(t))^2 \cdot e^{-\frac{\Delta t}{T_3}} \cdot \left( 1 - e^{-\frac{T_L - \Delta t}{T_3}} \right) + (l_2(t))^3 \cdot \left( 1 - e^{-\frac{T_L - \Delta t}{T_3}} \right) \right]. \]

Equation 3-81

Let \( T_L \gg T_3 \), and \( T_L - \Delta t \gg T_3 \), then

\[ F \propto \langle N_3(t) \rangle \approx \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot T_3 \cdot T_L^2 \]

\[ \cdot \left( (l_1(t))^3 + 3 \cdot (l_1(t))^2 \cdot (l_2(t)) \cdot e^{-\frac{\Delta t}{T_3}} + 3 \cdot (l_1(t)) \cdot (l_2(t))^2 \cdot e^{-\frac{\Delta t}{T_3}} + (l_2(t))^3 \right). \]

Equation 3-82

If we block the orange path, \( l_2(t) = 0 \), then

\[ \langle N_3^1 \rangle = \frac{1}{6} \cdot R_{23} \cdot R_{12} \cdot R_{01} \cdot N_0 \cdot T_3 \cdot T_L^2 \cdot \langle l_1(t) \rangle^3. \]

Equation 3-83
Equation 3-83 is consistent with our previous work (Equation 3-42 when $T_l \gg T_3$).

Use Equation 3-82 divide by Equation 3-83, we have

$$\frac{F_3^{1\&2}}{F_3^1} = \frac{\langle N_3^{1\&2} \rangle}{\langle N_3^1 \rangle}$$

$$= \frac{\langle I_1(t) \rangle^3 + 3 \cdot \langle I_1(t) \rangle^2 \cdot \langle I_2(t) \rangle \cdot e^{-\frac{At}{\tau_1}} + 3 \cdot \langle I_1(t) \rangle \cdot \langle I_2(t) \rangle^2 \cdot e^{-\frac{At}{\tau_2}} + \langle I_2(t) \rangle^3}{\langle I_1(t) \rangle^3}.$$  

Equation 3-84

Therefore, by varying $I_2(t)$ using the neutral density filter and meanwhile monitoring the fluorescence signal, we can solve $T_1$ and $T_2$, which are the lifetime of the intermediate states.

This experiment will provide more precision on the lifetime of the intermediate states compare to our previous estimate.

This experiment was proposed but not completed due to the lack of equipment.

### 3.3 Samples

The eumelanin sample from sepia (M2649-100MG, Sigma-Aldrich) and synthetic eumelanin sample (M8631-100MG, Sigma-Aldrich) were purchased. To prepare for imaging, 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.

When testing samples in nitrogen, the samples were mounted inside culture dishes so that the air could be purged with nitrogen. To prevent dehydration of the sample, 100% relative humidity on the sample was maintained by constantly running nitrogen bubbles through water.
The transmission of the culture dishes was tested and equal to that of the cover glasses used in all experiments in this work. The activation threshold as well as the signal strength of synthetic melanin is tested to be equal in dry nitrogen and humid nitrogen, therefore, we believe that the humidity does not affect the test results.

The iron-removed melanin and iron-saturated melanin were obtained from Warren S. Warren’s group at Department of Chemistry, Duke University, Durham, North Carolina, USA (50). The iron-removed melanin was originally sepia melanin purchased from Sigma-Aldrich and then washed with EDTA to remove metals. A portion of the EDTA-washed melanin was then saturated with iron (III) chloride. Iron content was measured with inductively coupled plasma mass spectrometry (ICP-MS). The iron-saturated melanin contained 27 494 ppm iron, and the EDTA-washed eumelanin contained only 30 ppm iron (50, 52).

The black human hair and white human hair samples were from a 27 year-old man. The blonde human hair sample was from a 31-year-old man.

All samples were either placed between a cover glass (No.1 1/2, Corning) and a microscope slide (12-550A, Fisherbrand) or inside a culture dish filled by nitrogen. The transmission of the cultures dish and the cover glass was tested to be equal.

Other biological samples used in this work will be introduced later.

3.4 Activation

Activation is crucial in obtaining melanin SMPAF. No melanin SMPAF can be obtained without the activation process. Therefore, study of the activation process provides us valuable information on understanding the photophysics of melanin SMPAF.
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 $10^5 \sim 10^6 \, W/cm^2$. Details of the activation threshold will be discussed later. After activation, SMPAF signals can be detected below the activation threshold. Therefore, the laser power is usually turned down after activation to protect samples from damages caused by high laser power. Melanin SMPAF can be activated and excited equally well using pulsed and CW laser(44). The activation time varies among melanin particles, but is usually less than 60 s. The average activation time decreases when the laser power increases.

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, which will be discussed in Chapter 3.9.1.

The activation process is irreversible. An experiment has been performed to activate synthetic melanin (M8631-100MG, Sigma) using a 920 nm pulsed laser. The activation threshold of the sample is $1.9 \cdot 10^5 \, W/cm^2$ in air. The sample was activated under $7.9 \cdot 10^5 \, W/cm^2$ laser intensity and imaged under $1.1 \cdot 10^5 \, W/cm^2$. The same signal strength was observed after four days using $1.1 \cdot 10^5 \, W/cm^2$ intensity.

### 3.4.1 Activation threshold

The tested activation threshold of various types of melanin is compared in Figure 3-4. The activation threshold is determined by increasing the input laser power step by step until SMPAF signals have been detected. At least 1 min is waited at each step.
Figure 3-4. Comparison of melanin SMPAF activation threshold in air and nitrogen.

From Figure 3-4, we can see that the activation threshold varies according to the type of melanin as well as the surrounding environment. Generally, the activation threshold is high when melanin is immersed in water. 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 (50). The data may indicate that iron plays a role in the activation of the melanin. However, as we can obtain similar signal strength in EDTA-washed melanin and in iron-saturated melanin after activation, the activation process may be more complicated than what Simpson suggested.
3.4.2 Melanin EPR signals

Electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR), detects unpaired electrons in the sample (53). EPR spectroscopy, the technology to study free radicals (54), has been used to investigate melanin (19, 54-57). Previous study has indicated that transient melanin radicals can be induced by light (58). We hereby present a preliminary study of comparing melanin EPR signals before and after activation to further understand the activation process (Figure 3-5).

The sample, sepia melanin with ~ 7 mm diameter and 0.12 mm thickness, was mounted between cover slides. The melanin ESR measurements were performed on the Bruker EMX EPR Spectrometer. The same sample was then activated in air by 920 nm pulsed laser on 3DFM 2.0. A high laser intensity \((1.36 \cdot 10^6 \, W/cm^2)\) was used for activation to ensure that the deeper layer of melanin was activated. The EPR signal of the activated sample was measured by the same Bruker EMX EPR Spectrometer.
Figure 3-5(a) shows that the intensity of the melanin EPR signal was decreased after activation. The positive peak of the EPR signal decreased by 54.24% after activation, and the negative peak of the EPR signal decreased by 50.75% after activation. The shapes of the EPR signals remained the same after activation (Figure 3-5(b)).

The comparison of melanin EPR signals before and after activation indicates that free radicals have been removed from melanin during the activation process. This result gives us more clues on understanding the activation process.
3.5 Specificity

Figure 3-6. Multimodal images of a white hair and a black hair from the same person. SMPAF image demonstrates very high specificity. (a) Brightfield. (b) CRM. (c) MPFM. (d) SMPAF. $\lambda_{\text{exc}} = 785$ nm.

The specificity of melanin SMPAF is higher compared to the traditional MPFM excited by a femtosecond pulsed laser. Most biological components emit auto-fluorescence under a pulsed laser, which causes background signals. In contrast, among tested biological components in skin till now, melanin is the only component that emits SMPAF signals under the activation process. Therefore, melanin SMPAF activated and excited using a CW laser demonstrates high specificity, which leads to numerous applications of melanin SMPAF imaging.
Figure 3-6 shows multimodal images of a white hair and a black hair from the same person. The white hair and the black hair can be easily distinguished from the brightfield image (Figure 3-6(a)). However, we were not able to locate melanin in the image. The CRM image provides more detailed physical structure of both hairs, but again, melanin is not distinguishable from the image (Figure 3-6(b)). The MPFM image shows both melanin SMPAF signals and background signals from other biological components (Figure 3-6(c)). The SMPAF image activated and excited using a CW laser demonstrates high specificity and is background-free. Figure 3-6 proves the feasibility of SMPAF imaging on detection of melanin inside biological samples.

3.6 Spectra

3.6.1 Spectra measured under 3DFM

The old 3DFM was used to measure the melanin SMPAF spectra in the region of 450 ~700 nm.

3.6.1.1 SMPAF spectra of sepia melanin

![SMPAF spectra of sepia melanin](image.png)

Figure 3-7. Excitation source dependence of sepia melanin SMPAF spectrum. The shapes of the fluorescence spectra remained exponential under different excitation source.
The SMPAF spectra of sepia melanin under various types of excitation sources are shown in Figure 3-7. The shapes of the fluorescence spectra remained exponentially distributed under four different excitation sources: 830 nm CW, 830 nm pulsed, 920 nm CW, 920 nm pulsed.

The time dependence of SMPAF spectra of sepia melanin was also studied (Figure 3-8). As time increased (t_{red} > t_{yellow} > t_{blue} > t_{green}), the intensity of the fluorescence signal decreased (Figure 3-8(a)), but the spectra remained the same shape when normalized (Figure 3-8(b)). This indicates that no new emissive components were created as the melanin was photo-bleached.

Figure 3-8. Time dependence of SMPAF spectra of sepia melanin. (a) As time increased, the intensity of fluorescence signal decreased (t_{red} > t_{yellow} > t_{blue} > t_{green}). (b) The spectra remained the same shape when normalized. λ_{exc} = 830 nm CW.
Although most spectra follow an exponential distribution with wavelength (blue curve in Figure 3-9(a)), there was a substantial minority (35.71%) that had a different shape (red curve in Figure 3-9(a)). It is clear that there is an additional peak at about 525 nm for the minority of our observations. We subtracted the two curves to obtain Figure 3-9(b). The spectrum in Figure 3-9(b) peaks at around 525 nm, and is very similar to the SMPEF spectrum of melanin reported previously (42, 43). Therefore, we believe that the red curve in Figure 3-9(a) is a mixed spectrum of SMPEF spectrum and SMPAF spectrum. However the SMPEF spectrum is relatively a small component of the integrated fluorescence signal.

3.6.1.2 Melanin SMPAF spectra in tissue

Fluorescence-spectra of melanin in different tissue are shown in Table 3-1. The first column contains the names of the samples. The second column shows the SMPAF spectra of the corresponding samples. The third shows the mixed spectra of both SMPEF spectra and SMPAF spectra. Human hairs of different colors were also compared. Yellow curves are melanin fluorescence spectra from blonde hair; black curves are melanin fluorescence spectra from black hair; the green curve is the melanin fluorescence spectrum from white hair. The rat skin and hair
samples were from a laboratory rat sacrificed in unrelated research and the zebra fish samples were 7-day-old larvae.

Table 3-1. Fluorescence spectra of melanin in tissue

<table>
<thead>
<tr>
<th>Sample</th>
<th>SMPAF spectra</th>
<th>Mixed spectra</th>
<th>Percentage of Mixed Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Hair</td>
<td><img src="image1" alt="SMPAF spectra" /></td>
<td><img src="image2" alt="Mixed spectra" /></td>
<td>32.74%</td>
</tr>
<tr>
<td></td>
<td>( \lambda_{exc} = 920 \text{ nm CW} )</td>
<td>( \lambda_{exc} = 920 \text{ nm CW} )</td>
<td></td>
</tr>
<tr>
<td>Rat Hair</td>
<td><img src="image3" alt="SMPAF spectra" /></td>
<td><img src="image4" alt="Mixed spectra" /></td>
<td>38.46%</td>
</tr>
<tr>
<td></td>
<td>( \lambda_{exc} = 920 \text{ nm CW} )</td>
<td>( \lambda_{exc} = 920 \text{ nm pulsed} )</td>
<td></td>
</tr>
<tr>
<td>Rat Skin</td>
<td><img src="image5" alt="SMPAF spectra" /></td>
<td><img src="image6" alt="Mixed spectra" /></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>( \lambda_{exc} = 920 \text{ nm pulsed} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iris of Zebra Fish</td>
<td><img src="image7" alt="SMPAF spectra" /></td>
<td><img src="image8" alt="Mixed spectra" /></td>
<td>73.91%</td>
</tr>
<tr>
<td></td>
<td>( \lambda_{exc} = 830 \text{ nm CW} )</td>
<td>( \lambda_{exc} = 830 \text{ nm CW} )</td>
<td></td>
</tr>
</tbody>
</table>
3.6.1.3 Mixed spectra decay to SMPAF spectra with time

![Graph showing mixed spectra decay with time for sepia and hair melanin](image)

Figure 3-10. (a) Time dependence of mixed spectra of sepia melanin. \( t_{\text{green}} > t_{\text{red}} > t_{\text{blue}} \) \( \lambda_{\text{exc}} = 920 \text{ nm CW} \). (b) Time dependence of mixed spectra of hair melanin. \( t_{\text{black}} > t_{\text{red}} > t_{\text{blue}} \) \( \lambda_{\text{exc}} = 830 \text{ nm CW} \).

We also noticed that mixed spectra gradually decayed to SMPAF spectra when the laser exposure time increased (Figure 3-10). This decay indicates that the SMPEF spectrum photo-bleaches faster than the SMPAF spectrum.

3.6.1.4 Probabilities of generating mixed spectra

Excitation source dependence of the probabilities of generating mixed spectra from sepia melanin and human hair melanin were examined. Figure 3-11 shows that the probabilities of generating mixed spectra does not change significantly due to excitation laser mode (~ 33%), but the probabilities of generating mixed spectra were much higher when the excitation laser was 920 nm (50% in sepia, 39.34% in hair) than 830 nm (25% in both sepia and human hair).
3.6.1.5 Conclusion

The spectra of SMPAF followed exponential distribution with wavelength. There is a probability of about 33\% that previously shown SMPEF can also be generated using the same process as SMPAF. 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 SMPF spectra.

3.6.2 Peak of SMPAF spectrum

A separate experimental setup is employed for detecting the peak spectrum of melanin SMPAF (Figure 3-12).

A 1505.9 nm laser is utilized (6300-LN, Velocity) as the activation source. The monochromator (ACTON sp-2500i, Princeton Instrument) consists of a 300 groove grating and a liquid-nitrogen-cooled CCD (Spec-10:400B/LN, Princeton Instrument). The wavelength of the spectrometer is calibrated using the software provided by the manufacturer. The spectral
sensitivity of the spectrometer is calibrated using a tungsten light source at a predetermined temperature (Q6.6AT3, GE).

![Experimental setup for detection of the peak spectrum of melanin SMPAF.](image1)

Figure 3-12. Experimental setup for detection of the peak spectrum of melanin SMPAF.

![SMPAF spectrum of sepia melanin. λ_{exc} = 1505.9 nm CW.](image2)

Figure 3-13. SMPAF spectrum of sepia melanin. λ_{exc} = 1505.9 nm CW.

Figure 3-13 shows the corrected SMPAF spectrum of sepia melanin. The peak of the fluorescence spectrum is around 960 nm.
3.6.3 Discussion

The broad spectrum of melanin SMPAF at visible and NIR wavelengths activated by NIR laser reveals a new and low-cost method of melanin detection. The SMPAF spectrum peak at 960 nm shows that the majority of SMPAF photons locate at the wavelength of 940 nm ~ 980 nm. Detection of SMPAF signal near 960 nm can be a more energy efficient way of melanin detection. By lowering the incident photon energy, it is possible to detect melanin SMPAF and meanwhile avoid the damage of biological components due to high laser power. The peak of the spectrum of melanin SMPAF may also shift with the wavelength of excitation source or with the different kinds of melanin in different biological samples. Further work is needed to confirm the feasibility of the SMPAF in the peak region of the spectrum.

3.7 Photon number absorption

3.7.1 Background

The number of absorbed photons in a melanin SMPAF process reported in Kerimo’s article was three, with an excitation intensity of \( \sim 10^6 \text{ W/cm}^2 \) (5, 44).

Research in rare-earth materials has shown that an anomalous power dependence is possible when the excitation laser intensity exceeds certain threshold (59, 60). This anomalous power dependence can be either due to a closed positive looping upconversion (CPLUC) mechanism or photon avalanche (PA). Both mechanisms cause an increase in the number of photons absorbed. In both cases, the number of photons absorbed is not predictable and can be an arbitrary number (59, 60). Here we report similar anomalous power dependence for melanin at a higher laser intensity.
3.7.2 Theory

The power dependence curve (PDC) of the fluorescence can be described using the equation below:

\[ F \propto A \cdot P^N, \]

Equation 3-85

where \( F \) is the fluorescence signal, \( A \) is the number of particles activated, \( N \) is the number of photons absorbed, and \( P \) is the input laser power. Taking the logarithm of both sides of Equation 3-85,

\[ \log(F) \propto \log(A) + N \cdot \log(P), \]

Equation 3-86

where \( N \) is the slope of the PDC in log scale. *e.g.* for a three-photon absorption \((N=3)\), the PDC is a cubic curve. The slope of the PDC in log scale is three. \( N \) can be a non-integer for complex processes (*e.g.* a mixture of second-order and third-order process will result in an \( N \) that is \( 2 < N < 3 \)).
3.7.3 Photon number absorption of melanin in mouse hair

Figure 3-14 shows a typical PDC of the melanin SMPAF in mouse hair. The red curve is the polynomial fit of fluorescence intensity vs. incident power. The activation source is a 920 nm pulsed laser. The filter used is the broadband filter (370 nm ~ 650 nm). The polynomial fit equation is:

\[ F = 5.99p^3 + 24.77p^2, \]

Equation 3-87
where \( F \) is the fluorescence signal, \( P \) is the input laser power. The PDC does not fit into a polynomial curve with an order higher than three. The polynomial fit curve with second and third-order indicates that a two-photon process is mixed with a third-order process.

This result suggests that the fluorescence signal detected is a signal of SMPAF observed previously mixed with a second-order process, which might be the SMPEF detected by Teuchner and Hoffmann. There is also a possibility that the procedures of generating melanin SMPAF can also generate a second-order process in the mouse hair. Further study is needed to determine the number of photons absorbed in the activation procedure.

### 3.7.4 Photon number absorption of sepia melanin when activated using high laser power

In this work the activation laser intensity is \( \sim 2 \cdot 10^6 \text{ W/cm}^2 \), which is over twice of the intensity we normally used, and 6 times of the activation threshold of sepia melanin in nitrogen. All sepia melanin samples in this work were immersed in nitrogen to minimize photo-bleaching.

Since the laser intensity used was strong, each illumination point was repeated at least twice in order to check for photo-bleaching. Unrepeateable illumination points were considered as photo-bleaching and therefore, discarded. A total of 28 valid illumination points were recorded on different melanin particles. Overall, we observed three types of PDC, which are discussed below.
3.7.4.1 Power dependence curve (PDC)

Figure 3-15. Three types of power dependence curves of sepia melanin when activated using high laser power.

Three types of PDCs of sepia melanin are shown on Figure 3-15. Figure 3-15(a) is a typical Type 1 PDC which shows the fluorescence signal when the laser intensity is scanned from low to high (tracks shown by green arrows: Point 1 to Point 2 and Point 2 to Point 3) and then immediately scanned from high to low (Point 3 to Point 4 and Point 4 to Point 1). As the laser intensity turned from low to high (Point 1 to Point 2), a new excitation channel was activated. In the typical curve shown here, $N_1 \approx 5.9$. After the laser intensity reached Point 2, the PMT was
saturated by the high emission. Then the laser intensity was turned down again (Point 3 to Point 4). In the resulting plot, $N_2 \approx 6.1$. Although the number of photons absorbed remained the same (meaning the slope of the curve unchanged), the intercept was different. This indicated that some of the activated melanin particles were photo-bleached. When the laser intensity decreased below Point 4, the emission became undetectable by the PMT.

Figure 3-15(b) shows a typical Type 2 PDC. When the laser intensity started below a threshold ($I_{th}$) and was turned from low to high, $N$ remained approximately $N \approx 1$. The path on the plot is represented by the blue points. This behavior indicated a one-photon absorption process. After the power reached $I_{th}$, $N$ increased suddenly to $N = 4.4$. However, if the laser intensity was turned from high to low at the same illumination point, represented on the plot by the green points, the PDC didn’t retrace above $I_{th}$, but retraced below $I_{th}$. The number of photons absorbed while the power remained greater than $I_{th}$ was $N = 6$, different than when the laser intensity was scanned from low to high. Therefore, two different processes must occur in this region. However, $N$ was consistent below $I_{th}$.

Figure 3-15(c) shows a typical Type 3 PDC. When the laser intensity was turned from low to high (blue points), $N \approx 1$ while the laser intensity was less than $I_{th1}$, which was similar to Type 2. But as the laser intensity increased, $N$ stayed close to $N \approx 0$. When the laser intensity was greater than $I_{th2}$, $N$ increased dramatically. But if the laser intensity was turned from high to low (green points), the PDC didn’t retrace above $I_{th2}$, but retraced below $I_{th2}$. Again, the number of photons absorbed, $N$, with the laser intensity greater than $I_{th2}$ might not be consistent when the same process was repeated at the same illumination point. However, $N$ was consistent with the laser intensity less than $I_{th2}$.
3.7.4.2 Statistical analysis of experimental data

Among the 28 valid illumination points examined, 5 (17.86%) of them showed Type 1 PDC, 14 (50.00%) followed Type 2 PDC, and 9 (32.14%) demonstrated Type 3 PDC.

The numbers of photons absorbed in all illumination points are grouped into three different types as shown in Figure 3-16.

![Figure 3-16. Number of photons absorbed in each type.](image)

Figure 3-16(a) is the plot of N for Type 1. Here, the photons absorbed for all points were close to 6. The average number of photons absorbed for Type 1 was 6.1. Figure 3-16(b) is the plot of N for Type 2 and Type 3. Most Ns in Type 2 were close to 1, except two points where Ns were close to 3.5. The average of N for Type 2 was 1.2. All N₁, where the power was less than \( I_{th1} \) in Type 3 were close to 1. The average of N₁ in Type 2 was 0.73. All N₂, where the laser intensity was between \( I_{th1} \) and \( I_{th2} \) in Type 3, were close to 0.5. The average of N₂ in Type 3 was 0.46.

The thresholds of all illumination points are again grouped into the three different types, and plotted in Figure 3-17. The thresholds \( I_{th} \) for all points in Type 2 varied from \( 4 \cdot 10^5 \) W/cm²...
to $14 \cdot 10^5 \text{ W/cm}^2$. The average $I_{\text{th}}$ in Type 2 was $8.4 \cdot 10^5 \text{ W/cm}^2$. The thresholds of all points in Type 3 were more constant than those in Type 2. The average $I_{\text{th1}}$ in Type 3 was $2.6 \cdot 10^5 \text{ W/cm}^2$. The average $I_{\text{th2}}$ in Type 3 was $6.3 \cdot 10^5 \text{ W/cm}^2$.

![Thresholds of each type](image)

**Figure 3-17.** Thresholds of each type.

### 3.7.4.3 Discussion

Possible energy states in the three different types are shown in Figure 3-18.

![Possible energy states in the three different types](image)

**Figure 3-18.** Possible energy states in the three different types.
The PDC for Type 1 indicates that a new excitation process was activated, which is previously unheard of. This process could possibly be a 6-photon excited state absorption mechanism, or might result from electrons oscillating between states (Figure 3-18(a)).

The PDC for Type 2 could arise if electrons populate to State 1 after activation. Before the laser intensity reaches the threshold, transitions to State 2 may be induced by the absorption of one photon. Due to the fact that the wavelengths of emitted photons are shorter than the laser wavelength, State 1 should be a higher energy state than the ground state. After reaching the threshold, a possible CPLUC or PA may have occurred (Figure 3-18(b)).

In Type 3, the electrons populate to State 1 after activation. Before the laser intensity reaches the threshold, electrons are transferred to State 2, while transitions from State 2' to the ground state occur, resulting in a one-photon absorption. As the laser intensity increases, more and more electrons populate to State 2. At the same time, the number of electrons capable of transition from State 2' to the ground state is limited. Therefore, saturation of State 2' occurs. This explains why increasing the laser intensity resulted in negligible increase in emission (Figure 3-18(c)).

There are several possible causes for the three types of PDC. One explanation is that different energy states are activated. Another could be that different types of melanin produce different results when optical power is applied. This is likely true considering melanin is a mixture of oligomers, and differences in the aggregation could result in differences with regard to energy state distribution. Lastly, the variance could have resulted from the incident laser light being absorbed inhomogeneously, due to the uneven surface of melanin.
3.8 SMPAF imaging

3.8.1 SMPAF imaging of mouse hair and skin

The mouse hair and skin samples were from a black 6 strain laboratory mouse sacrificed for unrelated research. The samples were mounted inside culture dishes so that the air could be purged with nitrogen in order to reduce photo-bleaching. To prevent dehydration of the sample, 100% relative humidity on the sample was maintained by constantly running nitrogen bubbles through water.

The mouse hair and skin samples were imaged with 3DFM. The filters used in this work are a 460 nm filter (bandwidth 50nm), a 525 nm filter (bandwidth 40nm), a 620 nm filter (bandwidth 60nm) and a broadband filter (370 nm ~ 650 nm). The selection of filters corresponds to the broad melanin SMPAF spectra previously detected.
Figure 3-19. Images of a mouse hair at the same position using different imaging modalities and filters. The first four images show SMPAF using: a 460 nm filter (a), a 525 nm filter (b), a 620 nm filter (c) and a broadband filter (370 nm ~ 650 nm) (d). The activation source is a 920 nm CW laser. The broad distribution of the fluorescence signal activated by the CW laser matches the spectra of data we acquired previously, which confirms that the signals acquired are SMPAF images of melanin. (e) is a CRM image. (f) is a MPFM mode image using the broadband filter (370 nm ~ 650 nm) with activation being from a 920 nm pulsed (100 fs pulse width) laser. (g) is a composite image of (d) (red channel), (e) (blue channel), and (f) (green channel). The red channel shows the location of melanin SMPAF, the blue channel shows the physical structure of mouse hair, and the green channel shows the auto-fluorescence of hair components as well as SMPAF of melanin. (h) is the composite image of (d) (red channel) and (e) (blue channel). By combining CRM with SMPAF, we can locate the melanin, with respect to other hair components.

Figure 3-19 shows images of a mouse hair at the same position using different imaging modalities and filters. The first four images show SMPAF using: a 460 nm filter (a), a 525 nm filter (b), a 620 nm filter (c) and a broadband filter (370 nm ~ 650 nm) (d). The activation source is a 920 nm CW laser. The broad distribution of the fluorescence signal activated by the CW laser matches the spectra of data we acquired previously (5), which confirms that the signals acquired are SMPAF images of melanin. Figure 3-19(e) is a CRM image. Figure 3-19(f) is a MPFM mode image using the broadband filter (370 nm ~ 650 nm) with activation being from a 920 nm pulsed (100 fs pulse width) laser. Figure 3-19(g) is a composite image of Figure 3-19(d) (red channel), Figure 3-19(e) (blue channel), and Figure 3-19(f) (green channel). The red channel shows the location of melanin SMPAF; the blue channel shows the physical structure of the mouse hair; and the green channel shows the auto-fluorescence of hair components as well as
SMPAF of melanin. Figure 3-19(h) is the composite image of Figure 3-19(d) (red channel) and Figure 3-19(e) (blue channel). By combining CRM with SMPAF, we can locate the melanin, with respect to other hair components.

The CW laser prevents the images in MPFM mode from being affected by the auto-fluorescence signal from other hair components. Therefore the signal-noise-ratio (SNR) of the image is greatly increased, such that melanin is more easily detected and located. For example, the femtosecond pulsed laser generates background interference due to the multi-photon absorption by keratin (Figure 3-19(f)) and the CW laser image lacks this background (Figure 3-19(a)-(d)).

The background interference was even greater when imaging mouse skin with the pulsed laser. Figure 3-20 shows images of mouse skin at the same position using different imaging modalities. Figure 3-20(a) was collected using the broadband filter (370 nm ~ 650 nm) and CW laser at 920 nm. Figure 3-20(b) is the corresponding CRM image. Figure 3-20(c) is a multi-photon fluorescence image using the broadband filter (370 nm ~ 650 nm) activated by the 920 nm pulsed laser. It is clear that the background auto-fluorescence is very high with the pulsed laser and is not present in the CW laser image. Figure 3-20(d) is a composite image of Figure 3-20(a) (red channel) and Figure 3-20(b) (blue channel). The red channel shows the location of melanin, and the blue channel shows the physical structure of mouse skin.
Figure 3-20. images of mouse skin at the same position using different imaging modalities. The image in Figure 5(a) was collected using the broadband filter (370 nm ~ 650 nm) and CW laser at 920 nm. Figure 5(b) is the corresponding CRM image. Figure 5(c) is a multi-photon fluorescence image using the broadband filter (370 nm ~ 650 nm) activated by the 920 nm pulsed laser. It is clear that the background auto-fluorescence is very high with the pulsed laser and is not present in the CW laser image. Figure 5(d) is a composite image of Figure 5(a) (red channel) and Figure 5(b) (blue channel). The red channel shows the location of melanin SMPAF, and the blue channel shows the physical structure of mouse skin.

3.8.2 SMPAF imaging of mouse melanoma

3.8.2.1 Introduction

Malignant melanoma, the most aggressive skin cancer, is a malignant proliferation of melanocytes (61). Early visual detection of melanoma is critical for melanoma management (41). The current clinical diagnosis method is lengthy and painful, and the histopathologic diagnosis varies depending on the expertise of the dermatopathologist (62).
3.8.2.2 Samples

B6.Cg-Braftm1Mmcm Ptentm1Hwu Tg (Tyr-cre/ERT2)13Bos/BosJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under defined conditions in accordance with the institutional guidelines. Approximately 25% of mice developed skin melanomas spontaneously and were euthanized. Skin tumors were dissected and fixed in formalin overnight before being stored in 70% ethanol at room temperature.

Spontaneous cutaneous melanoma was excised from the mouse and divided into three fractions (one with 50% of the total tumor volume and the other two with 25% of the total volume each) along the red lines in Figure 3-21(a). One fraction of the tumor (25% of the total...
volume) was imaged from the side shown in Figure 3-21(b). Tumor pathology was documented with another fraction (50% of the total volume) and photographed using a 2X objective (Figure 3-21(c)) and a 20X objective (Figure 3-21(d)).

The formalin (Catalog # SF98-4) with 4% formaldehyde (W/V) and 1% methanol (W/V) was purchased from Fisher Scientific (Waltham, MA). The 70% ethanol was prepared by mixing 100% ethanol with deionized water.

All samples were imaged with 3DFM. For melanoma imaging, a large photon density (in the present work is $\sim 6 \cdot 10^6 \text{W/cm}^2$) was used to activate and image melanin SMPAF.

3.8.2.3 Results
Figure 3-22. Melanoma images with different modalities at the same location. a) Brightfield, b) MPFM, c) CRM, d) SMPAF using the broadband filter (370 nm – 650 nm), and e) composite of CRM (blue channel) and SMPAF (green channel). f) SMPAF using the 620 nm filter; g) SMPAF using the 525 nm filter; h) SMPAF using the 460 nm filter.

Note that the images of (b) - (h) were registered. (a) was imaged at the same location as (b) - (e).
Figure 3-23. Melanoma images with different modalities at the same location. (a) Brightfield, (b) MPFM, (c) CRM, (d) SMPAF using the broadband filter (370 nm ~ 650 nm), and (e) composite of CRM (blue channel) and SMPAF (green channel), (f) zoomed-in image of the circled area of (e). (f) demonstrates that SMPAF was able to detect melanin with very small amount which was not detectable by CRM. Note that the images of (b) - (e) were registered. (a) was imaged at the same location as (b) - (e).
Two melanoma locations are shown under different modalities (Figure 3-22 and Figure 3-23). Figure 3-22(a) and Figure 3-23(a) are brightfield images, while Figure 3-22(b) and Figure 3-23(b) are traditional MPFM images. Melanin shows relatively strong MPFM signals mixed with SMPAF signals. The MPFM signals from melanin are not distinguishable from other biological components inside the skin. Figure 3-22(c) and Figure 3-23(c) are CRM images. Melanin appears bright in the CRM images, which is consistent with previous research (36). But again, the CRM signals from melanin are not distinguishable from other biological components inside skin. Figure 3-22(d) and Figure 3-23(d) are SMPAF images using the broadband filter (370 nm ~ 650 nm). The images are background-free as melanin is the only component inside skin that emits SMPAF signals. Figure 3-22(e) and Figure 3-23(e) are composite images of CRM (blue channel, from Figure 3-22(d) and Figure 3-23(d)) and SMPAF (green channel, from Figure 3-22(c) and Figure 3-23(c)). By combining CRM with SMPAF, melanin can be located with respect to other skin components. Figure 3-23(e) also demonstrates that SMPAF was able to detect melanin with single-pixel-size (in the present work 0.467 × 0.444 μm) which was not detectable by CRM modality (region within the red circle). Figure 3-23(f) is a zoomed-in image of the circled area of Figure 3-23(e).

Figure 3-22(f-h) are SMPAF images using the 620 nm filter, the 525 nm filter, and the 460 nm filter respectively. The broad distribution of the fluorescent signal activated by the CW laser matches the spectra of data acquired previously (45), which confirms that the signals acquired are SMPAF images of melanin. The images of Figure 3-22(b) - Figure 3-22(h) are registered. Figure 3-22(a) was imaged at the same location as Figure 3-22(b) - Figure 3-22(h). The images of Figure 3-23(b) - Figure 3-23(e) were registered. Figure 3-23(a) was imaged at the same
location as Figure 3-23(b) - Figure 3-23(e). The system background noise was subtracted in Figure 3-22(c) and Figure 3-23(c).

Figure 3-24. Z-stack composite images of the melanoma in Figure 3-23. The 0 um location is the same image as Figure 3-23(e).
Figure 3-24 shows z-stack composite images of the melanoma in Figure 3-23. The zero-um location is the same image as Figure 3-23(e). Figure 3-24 shows that the sectioning ability of SMPAF imaging is comparable to CRM.

3.8.2.4 Discussion

The melanin detected in Figure 3-22(c) and Figure 3-23(c) using SMPAF appeared to have lower density than brightfield images (Figure 3-22(a) and Figure 3-23(a)), traditional MPFM images (Figure 3-22(b) and Figure 3-23(b)), and CRM images (Figure 3-22(d) and Figure 3-23(d)). This could be due to any of the three possible reasons: 1) the brightfield modality is a transmission modality, which shows the stack of all layers, while SMPAF only shows the slice of one layer; 2) the SMPAF signal came only from activated melanin, while the traditional MPFM and CRM signals also came from surrounding biological components; 3) only a portion of melanin at the same image plane was activated, which is consistent with previous studies (5). The reason that only a portion of melanin at the same image plane was activated needs to be further explored.

3.8.2.5 Conclusion

SMPAF images of mouse melanoma were compared with conventional MPFM images and CRM images. SMPAF images provide greater specificity for melanin detection than MPFM images and CRM images. SMPAF images also demonstrate the potential to increase resolution for detecting small-size melanin through its single-pixel-size detection capability. Considering the high performance and low-cost feature of the technology, melanin SMPAF is a promising technology for imaging melanoma to assist diagnosis.
3.8.3 Selective activation of melanin in sepia skin

The sepia sample is a piece of squid skin purchased from a grocery store. The sample is placed between a cover glass and a microscope slide. The SMPAF of melanin is activated at the intensity of $3 \cdot 10^5 \, W/cm^2$.

The sepia skin sample is imaged on the Target system.

![Figure 3-25](image)

Figure 3-25. Selective activation of melanin in sepia skin. (a) Brightfiled. (b) Composite of CRM (blue) and SMPAF (green). Image performed on the Target system.

Figure 3-25(a) is a brightfield image of the sepia skin. The red circled-area corresponds to Figure 3-25(b), which is a composite image of CRM (blue channel) and SMPAF (green channel). The melanin block in the CRM image of Figure 3-25(b) appears brighter than other components, which is consistent with previous report (36). The letters, “ZL”, the initials of the author’s name, are activated and imaged on the melanin block using SMPAF.
3.8.4 *in vivo* SMPAF imaging in human skin

3.8.4.1 Materials and methods

The images were collected from a 20-year old man with skin type IV. The imaged skin sites were from the forearms (Figure 3-26(a)) and the thenar eminence (Figure 3-26(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.

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$^2$.

Figure 3-26. The skin sites imaged in this work (red arrow).
3.8.4.2 Results

Figure 3-27. Composite of *in vivo* human skin images. Blue: CRM. Green: SMPAF. (a) is an image of the surface of the forearm shown in Figure 3-26(a). (b) is an image of the non-nevus part of the thenar eminence surface in Figure 3-26(b). (c) and (d) are images of the nevus in the thenar eminence in Figure 3-26(b).

All the images in Figure 3-27 are shown as composite of CRM (blue), which represents the physical structure of the skin, and SMPAF (green), which represents melanin. Figure 3-27(a) is an image of the surface of the forearm shown in Figure 3-26(a). Figure 3-27(b) is an image of the normal skin of thenar eminence in Figure 3-26(b). Figure 3-27(c) and Figure 3-27(d) are images of the nevus in the thenar eminence in Figure 3-26(b). Figure 3-27(c) and Figure 3-27(d) contain significant higher numbers of green pixels than Figure 3-27(a) and Figure 3-27(b), which
suggests that higher concentration of melanin in the nevus is captured by the SMPAF imaging channel.

3.8.4.3 Discussion

This work demonstrates the feasibility of in vivo melanin detection using SMPAF in human skin. According to our previous research, not all melanin in the image plane can be activated (5). Therefore, it is possible that the melanin observed in the SMPAF mode does not represent all melanin in the skin. The photophysics and photochemistry of activated states needs to be investigated to explain the non-uniformity of the melanin activation.

3.9 Laser ablation of melanin

As mentioned previously, the activation of melanin requires relatively high photon-density. Photo-bleaching or damage of melanin may occur if the input laser power remains high for a long time. This work studies the damage of melanin at high laser power as well as the application of SMPAF guided laser ablation of melanin for laser treatment and cosmetic applications.

3.9.1 Ablation of melanin in mouse hair caused by long-time high-power laser exposure

3.9.1.1 Materials and methods

The mouse hair and skin samples were from a black 6 strain laboratory mouse sacrificed for unrelated research. The samples were mounted inside culture dishes so that the air could be purged with nitrogen in order to reduce photo-bleaching. To prevent dehydration of the sample, 100% relative humidity on the sample was maintained by constantly running nitrogen bubbles through water.

The samples were imaged on 3DFM.
Figure 3-28. Brightfield images of mouse hairs taken by the SPOT camera. The signal generated by SMPAF was strong enough that it was visible to eyesight alone. (a) is a brightfield image of the mouse hairs before laser activation. (b) is a brightfield image of the mouse hairs after laser exposure for 7 seconds. Within these two images, the orange circular areas are places where melanin is activated. (c) is an expanded sub-image on the orange circular area in (a). (d) is an expanded sub-image on the orange circular area in (a) after laser exposure for 60 seconds. There is an obvious change to the melanin in the area of the red circle. The mouse hair in Figure 3-28 was activated and exposed under an 830 nm pulsed laser. The laser intensity at the sample is \( \sim 4.63 \times 10^6 \text{ W/cm}^2 \).

Figure 3-28 shows brightfield images of mouse hairs taken by the SPOT camera (note that the SPOT camera used here replaced the Thorlabs camera in Figure 2-8). The signal generated by SMPAF was strong enough that it was visible to eyesight alone. Figure 3-28(a) is a brightfield image of the mouse hairs before laser activation. Figure 3-28(b) is a brightfield image of the mouse hairs after laser exposure for 7 seconds. Within these two figures, the orange circular
areas are places where melanin is activated. Figure 3-28(c) is an expanded sub-image on the orange circular area in Figure 3-28(a). Figure 3-28(d) is an expanded sub-image on the orange circular area in Figure 3-28(a) after laser exposure for 60 seconds. There is an obvious change to the melanin in the area of the red circle. The mouse hair in Figure 3-28 was activated and exposed under an 830 nm pulsed laser. The laser intensity at the sample is $\sim 4.63 \cdot 10^6 \text{ W/cm}^2$.

### 3.9.1.3 Discussion

The above result shows that it is possible to damage melanin at high laser power ($\sim 4.63 \cdot 10^6 \text{ W/cm}^2$) if the laser exposure time is long ($\sim 60$ s). Therefore, for imaging purposes, the laser power is usually turned down after activation to protect the sample.

### 3.9.2 SMPAF guided selective ablation of melanin in sepia skin

#### 3.9.2.1 Background

Selective photothermolysis, invented by Anderson et al. in 1983, utilized nanosecond-domain laser pulses to selectively ablate melanin and melanin-related cells (63, 64). This method has been widely used for treating melanin-related skin diseases since then.

Despite the effectiveness, the photothermolysis technology lacks the ability of targeting individual melanin particles, which limits the application of the technology in treating skin diseases in some sensitive regions, e.g. nevus of ota around eyes. The use of a pulsed laser also increases the cost of the treatment. Therefore, it is important to develop a technology that is able to selectively ablate melanin with high precision.

#### 3.9.2.2 Samples and methods
The sepia sample is a piece of squid skin purchased from a grocery store. The sample is placed between a cover glass and a microscope slide. This work is performed on the Target system. A 10x 0.3NA objective is used for imaging.

A three-step procedure is followed for ablation of melanin:

Step 1, detection: the laser scans the designated area and meanwhile detects SMPAF.

Step 2, ablation: the laser beam targets at a position where SMPAF signals were detected. SMPAF signal is monitored during the ablation.

Step 3, stop and continue: once the SMPAF signal is no longer detected, melanin ablation at current position is considered complete. The laser beam then moves to the next position for ablation.

3.9.2.3 Results

![Figure 3-29. Brightfield images of full ablation of a melanin block in sepia skin: (a) before ablation; (b) after ablation. The laser exposure area is circled in red. Laser power at the sample: 39.2 mW.](image-url)
Figure 3-29 shows brightfield images of full ablation of a melanin block in the image plane. Figure 3-29(a) shows an image of the melanin block before ablation. The circled area is the laser exposure area. Figure 3-29(b) is an image after ablation. Note that only the melanin in the image plane has been ablated. To ablate all melanin, the image plane needs to be moved vertically. The laser power at the sample in this work is estimated to be 39.2 mW.

Figure 3-30. Partial ablation of a melanin block: (a) brightfield image before ablation; (b) brightfield image after ablation, the laser exposure area is circled in red, a letter “z” is created by partially ablate melanin at the center; (c) zoomed-in image of the laser exposure area in (b). (d) SMPAF image after ablation. Laser power at the sample: 62.9 mW.
To demonstrate the ability of performing melanin ablation with micrometer resolution, partial ablation was performed on a melanin block and shown in Figure 3-30. Figure 3-30(a) is a brightfield image of the sepia skin before laser ablation. Figure 3-30(b) is a brightfield image of the same location after laser ablation. Figure 3-30(c) is a zoomed-in image of the circled area in Figure 3-30(b). Figure 3-30(d) is a SMPAF image of the circled area after ablation. A letter “z” with the size of 25 × 35 μm is ablated on the melanin block. The laser power at the sample is estimated to be 62.9 mW.

3.9.2.4 Discussion

This work has demonstrated that SMPAF guided laser ablation of melanin is able to selectively ablate melanin with micrometer precision. Compared to the traditional selective photothermolysis, which uses nanosecond pulsed lasers for melanin ablation, this method demonstrates higher precision, higher specificity, deeper penetration, and avoids the use of a pulsed laser, or other expensive optical components.

Therefore, the SMPAF guided laser ablation of melanin is not only a promising low-cost technology for treatment of melanin-related skin diseases, but is also an important technology for cosmetic applications.

3.10 Conclusion

The theoretical analysis of stepwise multi-photon excitation and commonly known simultaneous multi-photon excitation has been performed to demonstrate that stepwise multi-photon excitation occurs under either a pulsed laser or a CW laser. The lifetime of the intermediate states and the excited state of activated melanin is calculated. Based on our
calculation, the lifetime of the intermediate states is estimated to be $\sim 10^{-9}$ s. An experiment is proposed to measure the lifetime of the intermediates with more precision.

Activation is crucial in obtaining melanin SMPAF. No melanin SMPAF can be obtained without the activation process. The activation process has been proven to be irreversible. The activation threshold varies according to the types of melanin as well as the surrounding environment. Generally, the activation threshold is high when melanin is immersed in water. The activation threshold undergoes $\sim 10 - 20\%$ increase when melanin is immersed in nitrogen compared 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 (50). Experiments have been performed to prove that such statement is partially true. The fact that the intensity of melanin EPR signals decreases after activation indicates that free radicals have been removed from melanin during the activation process.

The SMPAF is unique in melanin among biological components in skin. Therefore, melanin SMPAF reveals high specificity in melanin detection. The specificity of melanin SMPAF is demonstrated by comparing brightfield, CRM, MPFM, and SMPAF images of a white hair and a black hair from the same person.

The spectra of various melanin samples have been demonstrated. The broad spectrum of melanin SMPAF at visible and NIR wavelengths activated by NIR laser reveals a new and low-cost method of melanin detection. The melanin SMPAF emission spectrum activated by 1505.9 nm laser light is measured and shows a peak at 960 nm. There is a probability of about 33% that previously shown SMPEF can also be generated using the same process. As excitation source at
920nm has a higher chance of generating SMPEF peaks at 525 nm than excitation source at 830nm, SMPEF spectra photo-bleach faster than SMPAF spectra.

The SMPAF observed by Kerimo is a third-order process. The power dependence curve of the mouse hair suggests that the fluorescence signal detected is a mixture of 3-order process and 2-order process, which can be a SMPAF signal observed previously mixed with a the SMPEF detected by Teuchner and Hoffmann. There is also a possibility that the procedures of generating melanin SMPAF can generate a second-order process in mouse hair. Three different excitation channels have been activated using laser intensity of $\sim 2 \cdot 10^6$ W/cm$^2$. From the three excitation channels, three types of PDC are generated. The possible reasons for generating the three PDC types are: 1st, different energy states have been activated; 2nd, the types of melanin are different, and therefore they react differently to the input laser power. Since melanin is a mixture of oligomers, different aggregation could result in differences in distribution between energy states; 3rd, the laser light was inhomogeneously absorbed, caused by an uneven melanin surface.

The background-free feature of melanin detection using SMPAF has proved to be useful in SMPAF image. The application of melanin SMPAF imaging for detection of melanin in mouse hair and skin, mouse melanoma, and human skin has been demonstrated. Selective activation of melanin in sepia skin has also been demonstrated. The acquired multimodal images confirmed that the melanin SMPAF imaging is reliable. 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. By combining CRM with SMPAF, we can locate the melanin with respect to other biological components. SMPAF images also demonstrate potential to increase sensitivity for detecting small size melanin through its single-pixel-size detection capability. 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 selective activation of melanin in sepia skin has not only demonstrated the high-precision of melanin SMPAF imaging, but also revealed a new method for laser treatment of melanin-related diseases.

The amount of activated melanin increases with incident power and exposure time, but increased exposure causes a risk of photo-bleaching and instability. The damage of melanin caused by long-time high-power laser exposure has been demonstrated. Therefore, for imaging purposes, the input laser power needs to be reduced after melanin has been activated.

SMPAF guided laser ablation of melanin demonstrates the ability to selectively ablate melanin with micrometer precision. The ability to provide precise localized damage indicates that deeper penetration without collateral damage can be achieved by increasing the laser power. This work does not involve the use of a pulsed laser or other expensive optical components. Therefore, the SMPAF guided laser ablation of melanin is not only a promising low-cost technology for treating melanin-related skin diseases, but also an important technology for both medical and cosmetic applications.
4 Customized-Profile Lenses (CPL) for On-axis Laser Scanners

4.1 Introduction

Many applications in the fields of science and engineering have the need for a device that is able to control the deflection of a beam of light. The applications requiring such devices spread across many areas including defense, optical communication, aircraft and aerospace systems, graphic displays, imaging systems, materials processing etc. Currently, galvo mirrors, and polygon mirrors are widely used beam steering techniques. However, these approaches are off-axis solutions, which increase the size, error, and complexity of the overall system. Some on-axis approaches, such as acousto-optic deflectors (65-68) and electro-optic deflectors (69-73), are highly expensive. A relatively low-cost on-axis solution is the Risley Prisms based system (74-76), which creates complicated scanning patterns but lacks the ability of performing a raster scan.

Three customized-profile lenses (CPL) for on-axis laser scanners were designed for low-cost linear-motion scanners (77), low-cost fast spinning scanners (78), and high-precision fast spinning scanners, which will be introduced in Chapter 4.2, Chapter 4.3, and Chapter 4.4 respectively.

The original idea of customizing the lens profile for on-axis laser scanning was conceived by Dr. Yair Mega at the Optical Science Laboratory, Northeastern University. The CPL for a low-cost linear-motion scanner was designed by Dr. Mega and the author. The author then improved the original design by tailoring the concept to the industrial manufacturing ability and market demands and therefore invented the other two CPLs.
4.2 A CPL for low-cost linear-motion optical scanners

4.2.1 Principles

The principles of the CPL for low-cost linear-motion scanners are shown in Figure 4-1. The 3D layout of the CPL is shown in Figure 4-2. The incident side of the CPL is a flat surface. The other side of the surface is a customized-profile surface. As show in Figure 4-1, the CPL is
located at the solid line. The center of the incident plane is point O. The optical axis of the CPL is perpendicular to the incident plane and passes point O.

When the laser beam incident normally along y-axis at point O', the direction of the beam will not change. When the incident beam arrives at the other side of the CPL at point A, the beam will deflect and hit point B of the target. If originally the CPL is located at dotted line with incident plan centered at point O', the incident beam passes the optical axis of the CPL. The beam will not deflect, and will hit point B' of the target. The displacement of the beam at the target, B'B, is the deviation of the beam. O'O is the displacement of the CPL.

![Diagram of CPL with 3D layout and cross sections](image)

Figure 4-2. 3D layout of the CPL for low-cost linear-motion scanners

In order to create a linear scan, we need to make sure that the deviation of the beam at the target is proportional to the displacement of the CPL, which is:
From trigonometry:

\[ \frac{B'B}{AB} = \tan \beta, \]

Equation 4-2

where \( \beta \) is the deflection angle of the beam.

From Snell's law:

\[ n_1 \sin \theta_1 = n_2 \sin \theta_2, \]

Equation 4-3

where \( n_1, n_2 \) are the refraction index of the incident ray and the refracted ray. \( \theta_1, \theta_2 \) are the incident angle and the refracted angle.

We also have

\[ \frac{dy}{dx} = \tan \theta_1, \]

Equation 4-4

And

\[ \beta = \theta_1 + \theta_2. \]

Equation 4-5

By solving Equation 4-2 - Equation 4-5, we can get the profile of the CPL:
\[
y = \frac{1}{c_s} \cdot \frac{n_0}{n_1} \left\{ \sqrt{1 + (c_s \cdot x)^2} - 1 + \frac{n_0}{n_1} \cdot ln \left[ \sqrt{1 + (c_s \cdot x)^2} - \frac{n_0}{n_1} \right] \right\} + c_y,
\]

Equation 4-6

where \( x \) and \( y \) represent the profile of the CPL; \( c_s \) is a constant, which determines the shape of the CPL; \( c_y \) is a constant, which determines the thickness of the CPL in \( y \)-direction.

### 4.2.2 Design and manufacturing

![Figure 4-3. Photos of the CPL mounted on a linear motorized stage. (a) top view; (b) tilted top view.](image)

The CPL is designed for 1064 nm laser. The material of the CPL is BK7 glass, which has an index of refraction of 1.50669 at 1064 nm. The \( c_s \) of the CPL is \( 14.5 \, \text{m}^{-1} \). The CPL was mounted onto a linear motored stage (GCD-203100M, GCD-0301M, China Daheng Group, Inc.) for precise driving (Figure 4-3). A 1064nm CW laser was used (PGL-FS-1064, Changchun New Industries Optoelectronics Tech.) as a monochromatic light source. Two photographs of the described setup are shown in Figure 4-3. The curved profile of the proposed CPL can be seen in the figure. When the incident beam falls precisely at the center of the element, no deflection of the beam should be observed, while any shift of the element will result with a linear deflection to the displacement of the element.
4.2.3 Results

A measurement of the total displacement was taken on a screen located at 1 m from the exit surface of the CPL.

![Graph showing measured versus calculated deflection](image)

Figure 4-4. Measurement and calculation of the deviation as a function of the incident position.

Figure 4-4 depicts a measurement (shown in dotted black line) and the calculation (shown in dashed red) of the deviation as a function of the incident position. As can be easily seen in Figure 4-4, there is a good agreement between the expected deflection obtained analytically and the measured results. The experimental data showed a deviation of approximately $\Delta c_s = 0.07 \text{ m}^{-1}$ from the original design value with a standard deviation of $\sigma_{c_s} = 0.88 \text{ m}^{-1}$.

4.2.4 Discussion

The design of the CPL is tailored for mass-production and therefore is low-cost. The tested results demonstrate excellent linearity and match the designed parameters. The consistency between the tested results and design parameters indicates that the current design is suitable for high-precision applications, e.g. laser cutting systems, laser scanning imaging systems, etc.
4.3 A CPL for fast spinning on-axis optical scanners

4.3.1 Introduction

We have presented a CPL for low-cost linear-motion optical scanners, which can be easily manufactured and implemented to many optical systems. The steering apparatus combines refraction and linear mechanical movement. However, the previous CPL we presented requires a linear back-and-forth motion, which is a low-speed solution. We here introduce a CPL, tailored for high speed performance while maintaining the advantages of a linear response on-axis optical scan.

4.3.2 Principles

The principle of the CPL is described in Figure 4-5. The left surface of the CPL is a flat plane. The right surface of the CPL is a customized profile surface. The rotation axis of the CPL is along the z-axis (dotted line in Figure 4-5(a)). As shown in Figure 4-5(a), the incoming beam parallel to the rotation axis incidents normally to the left surface of the CPL. The direction of the beam does not change until reaching the right surface of the CPL. When the beam hits the right surface, the transmitted beam will deflect and hit point A at the target. If the CPL does not exist in the beam path, the direction of the beam will not change and the beam will hit point O at the target. OA is the projection of the transmitted beam in the target plane (xy plane). OA contains both x-component, \((OA)_x\), and y-component, \((OA)_y\) (Figure 4-5(b)). If the CPL is a prism, the trajectory of the beam at the target will be a circle when rotating the prism along the rotation axis, as the length of OA is constant (green dotted line in Figure 4-5(c)). To create a linear response optical scanner, point A needs to be moving along the orange dotted line on the target while the CPL is rotating at constant angular velocity along the rotation axis, which means \((OA)_x\) needs to be constant.
Figure 4-5 The principle of the CPL for fast spinning on-axis optical scanners.

We’ve modified the lens profile to follow the differential equation below:

\[
dy = \frac{C_1 \cdot r}{h \cdot \sqrt{r^2 - x^2}} \cdot dx, \quad -1 + \frac{n_1}{n_0} \sqrt{1 + \left(\frac{C_1}{h}\right)^2 \cdot \frac{r^2}{r^2 - x^2}}
\]

Equation 4-7

where \(C_1\) is a constant, which determines the shape of the CPL; \(r\) is the distance between the beam and the rotation axis. \(h\) is the distance between the CPL and the target. \(n_1\) is the refractive index of the CPL; \(n_0\) is the refractive index of the environment, in air, \(n_0 = 1\).
4.3.3 Design and manufacturing

The 3D layout of the CPL is described in Figure 4-6. The profile of the CPL is calculated using MATLAB. The CPL is designed for 1064 nm laser using BK7 glass ($n_1 = 1.50669$ at 1064 nm).

![Figure 4-6 3D layout of the CPL for fast spinning on-axis optical scanners.](image)

The CPL can be cut and spliced so that multiple scanning cycles can be achieved in one rotation. Here, we manufactured a four-cycle CPL scanner as an example (Figure 4-7).
Figure 4-7 The manufacturing process of a 4-cycle CPL for fast spinning on-axis optical scanners.

The xy-cross-section of the CPL is a rectangular shape (Figure 4-7(a)). After cutting the CPL along the dotted line in Figure 4-7(a), the xy-cross-section of the CPL is turned into a isosceles right triangle (Figure 4-7(b)). The CPL in Figure 4-7(b) is then cut along the dotted line in Figure 4-7(c). The process of Figure 4-7(a) - Figure 4-7(c) is repeated on four identical CPLs. Four identical blue parts of Figure 4-7(c) is obtained. When we splice the four blue parts of Figure 4-7(c) together with their right angles faced to the center, an octagon cross section is obtained (Figure 4-7(d)). This design creates a 4-cycle scan with each rotation. More cycles can be achieved in each rotation using a similar approach.
4.3.4 Results and discussion

A proof-of-concept prototype of a four-cycle CPL was manufactured (Figure 4-8). The CPL is driven by a motor (ZYTD-38SRZ-R, Zhengke Electromotor) with a spinning speed of 1800 RPM. The scanner creates a linear scan with 120 Hz. Higher scanning speed can be achieved with higher speed motors and more cycles of CPL. For example, a 32-cycle CPL mounted on a 12000 RPM motor will create a scanning speed of 6.4 kHz, which is enough for video-rate microscopy application.

This design is tailored for high speed performance while maintaining the advantages of a linear response on-axis optical scanner. The manufacturing process is simple and can be easily implemented for mass production, minimizing the manufacturing cost per device compared to other on-axis solutions.

Since the proposed device is an on-axis solution, when implementing the device into an optical system, the size, error, and complexity of the overall system will be reduced.
4.3.5 Conclusion

The functionality of a CPL based linear response fast spinning on-axis optical scanner is demonstrated. The proposed optical beam steering device is able to meet the needs for video-rate scanning. The manufacturing cost per device is low compared to other on-axis solutions.

The intuitiveness and ease of use makes the proposed device highly convenient to be implemented into laser scanning systems. The implementation of the CPLs in laser scanning systems is promising in improving the current 3D laser scanning microscopy systems by reducing the size, error, and complexity of the overall system. The device can also be applied to other systems unitizing high speed laser scanning technique including lasers cutting, and laser printing, etc.

4.4 A CPL for high-precision fast spinning optical scanners

4.4.1 Introduction

The CPL designed in Chapter 4.3 is high-speed and low-cost. However, as the spinning angle is not linear to the deviation, the speed of the laser beam at the target is not constant when the CPL is spinning at a constant angular velocity (Figure 4-9).
Figure 4-9. The deviation of the laser beam at the target as a function of spinning angle of the CPL designed in Chapter 4.3. Simulated using MATLAB.

The concept of this design is to convert the design of CPL for low-cost linear-motion optical scanners (described in Chapter 4.2) from xy-coordinate into cylindrical-coordinate to create a CPL for high-speed, high-precision optical scanners.
4.4.2 Principle

Figure 4-10. Principles of the CPL for high-precision fast spinning optical scanners.

(a) Design principles (3D).

(b) Target plane (xy plane).
The principle of the CPL is described in Figure 4-10. The 3D layout of the CPL is shown in Figure 4-11. As shown in Figure 4-10(a), the incoming beam incident from the bottom surface of the CPL. The transmitted beam will hit point C at the target. Figure 4-10(b) shows the projection of transmitted beam in xy plane (target plane) as B’C. To create a linear scan, we need to have the y-component of B’C, CC_y, proportional to the spinning angle \( \phi \). Similar to the CPL described in Chapter 4.2, we need to have
\[
\frac{CC_x}{BB'} = \tan \beta \propto \varphi.
\]

Equation 4-8

Similar to the design of the CPL described in Chapter 4.2, by solving the equations in cylindrical-coordinate, we can get

\[
z = \frac{\rho}{c_s} \cdot \frac{n_0}{n_1} \left( \sqrt{1 + (c_s \cdot \varphi)^2} - 1 + \frac{n_0}{n_1} \cdot \ln \left( \frac{\sqrt{1 + (c_s \cdot \varphi)^2} - \frac{n_0}{n_1}}{1 - \frac{n_0}{n_1}} \right) \right) + c_z,
\]

Equation 4-9

where \(z\) and \(\rho\) represent the profile of the CPL; \(c_s\) is a constant, which determines the shape of the CPL; \(c_z\) is a constant, which determines the thickness of the CPL in \(z\)-direction.

Similar to the CPL for fast spinning on-axis optical scanners described in Chapter 4.3, the scanning speed can be multiplied by implementing multiple cycles. Figure 4-10 and Figure 4-11 are based on a 4-cycle CPL.

### 4.4.3 Design and manufacturing

Unlike the two CPLs described previously, which used BK7 glass for manufacturing, this CPL is made of PMMA, with index of refraction 1.467 at 1060 nm. The choice of the material is based on the industrial manufacturing ability. The inner radius of the CPL is 5 mm, and the outer radius of the CPL is 25 mm.

### 4.4.4 Results

The deviation of the laser beam at the target as a function of spinning angle of the CPL has been simulated by MATLAB and shown in Figure 4-12. The simulated results show that the
deviation of the beam is linear to the spinning angle. Therefore, the scanning speed of the CPL is constant the CPL is spinning at constant angular velocity.

![Graph showing beam deflection vs. spinning angle](image)

**Figure 4-12.** The deviation of the laser beam at the target as a function of spinning angle of the CPL. Simulated using MATLAB.

### 4.4.5 Discussion

The linear response of the beam deflection versus the spinning angle indicates that this design can exhibit very high precision when running at a high speed.

### 4.5 Conclusion

Three CPLs for different applications are designed, two of which are manufactured and tested. The other one has been proved by simulation. The CPLs designed for beam steering devices are powerful tools for laser cutting, 3D printing, 3D laser scanning microscopy, *etc.*
5 Conclusion

3DFM has been upgraded to 3DFM 2.0 with improved performance and flexibility. The Target system has been built for targeting small particles with micrometer resolution. The control systems developed for both 3DFM 2.0 and the Target system are low-cost and easy-to-build, with all components off-the-shelf. The system has not only significantly decreased the complexity and size of the microscope, but also increased the pixel resolution and flexibility of the microscope. The development of both systems has fostered biomedical research, especially the study of melanin SMPAF.

Theoretical analysis on the excitation process of SMPAF has been performed. The spectra, activation threshold, and photon number absorption of melanin SMPAF have been investigated. The above studies provide clues in understanding the photophysics of melanin SMPAF.

SMPAF images of melanin in mouse hair and skin, and mouse melanoma, are compared with images taken by conventional MPFM and CRM. SMPAF images significantly increase specificity for melanin detection compared to MPFM images and CRM images. SMPAF images also demonstrate the potential to increase sensitivity for detecting small-size melanin granules. The application of melanin SMPAF imaging in detecting melanin inside human skin in vivo has also been demonstrated.

Selective ablation of melanin in sepia skin with micrometer precision has been demonstrated on the Target system. Compared to the traditional selective photothermolysis, which uses nanosecond pulsed lasers for melanin ablation, this method demonstrates higher precision, higher specificity and deeper penetration. Therefore, the SMPAF guided selective ablation of melanin is a promising tool of removing melanin for both medical and cosmetic purposes.
Three CPLs for on-axis laser scanners have been designed for low-cost linear-motion scanners, low-cost fast spinning scanners, and high-precision fast spinning scanners. The CPLs for low-cost linear-motion scanners and low-cost fast spinning scanners have been manufactured and tested. The CPL for high-precision fast spinning scanners has been proved by simulation. The CPLs designed for beam steering devices are useful tools for laser cutting, 3D printing, 3D laser scanning microscopy, etc.

The development of CPLs can enhance the performance of laser-scanning modalities in multimodal microscopes and therefore further boost biomedical research, including the study of melanin SMPAF.
Bibliography