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Nonlinear Absorption Microscopy†

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Abstract

For the past two decades, nonlinear microscopy has been developed to overcome the scattering problem in thick tissue imaging. Owing to its increased imaging depth and high spatial resolution, nonlinear microscopy becomes the first choice for imaging living tissues. The use of nonlinear optical effects not only facilitates the signal originating from an extremely small volume defined by light focusing but also provides novel contrast mechanisms with molecular specificity. Nonlinear absorption is a nonlinear optical effect in which the absorption coefficient depends on excitation intensity. As a commonly used spectroscopy tool, nonlinear absorption measurement uncovers many photophysical and photochemical processes correlated with electronic states of molecules. Recently we have been focusing on adapting this spectroscopy method to a microscopy imaging technique. The effort leads to a novel modality in nonlinear microscopy—nonlinear absorption microscopy. This article summarizes the principles and instrumentation of this imaging technique and highlights some of the recent progress in applying it to imaging skin pigmentation and microvasculature under *ex vivo* or *in vivo* conditions.

INTRODUCTION

The primary use of optical microscopes is to visualize the small structures that cannot be resolved by the naked eye. Illumination in microscopic observation is indispensable. The images projected to our retina by the microscope optics are essentially the contrast patterns caused by nonuniform absorption or scattering properties of the specimen within the illuminated area. These are the images that we used to see in bright field or dark field microscopy. In addition, phase contrast and differential interference contrast microscopy are complementary techniques for producing high contrast images of transparent specimens by detecting the phase changes when light passes through the specimen. These contrast mechanisms have provided us magnificent structural information in submicron resolution, yet little about the underlying molecular constituents—the level at which we would like to understand biological functions or disease processes (1). So the molecular contrast microscopy has been developed to address the imaging needs that require localizing and

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quantifying different molecular constituents in the cellular or subcellular structures of interest (2), or visualizing and measuring dynamic biomolecular processes in micro compartments or functional organelles (3–5). Molecular contrast microscopy allows tracking gene expression and protein transportation, studying the dynamic protein-protein interactions and more importantly, studying how cells coordinate with each other to realize meaningful physiological functions. Molecules identify themselves spectroscopically; molecular contrasts in optical microscopy come naturally from optical spectroscopy. So the question becomes how to do spectroscopy while imaging at the same time. There are fundamentally two strategies to collect spectrally resolved images: acquiring images at different wavelengths (or band) sequentially and reconstruct spectra data for each image pixel, or acquiring the spectrum at a single spot and reconstruct the images pixel by pixel by scanning the spot. In reality the strategy chosen is a trade-off among several factors, such as image acquisition speed, spectral resolution, spatial resolution and imaging goals. The load of imaging work can be dramatically reduced if there is enough spectroscopy knowledge about the molecules. For example in fluorescence microscopy (6) dyes or fluorescent proteins are often used as probes that exclusively report the existence of specific molecules through labeling process and genetic expression. In those types of imaging applications, optical interference filters can be used in imaging the targeted molecules.

Optical microscopy of biological tissues, especially thick tissues, suffers one problem—light scattering, which causes photons deviating many times before they reach light sensors; as a result, blurred images are formed and the resolution of images is severely reduced when the subsurface of tissue is imaged. The general practice in microscopy uses a very thin layer of samples sectioned from the tissue to minimize the scattering effects; however, sectioning is apparently not practical for imaging living tissues in their intact conditions. In the past, nonlinear optical microscopy (7,8) was developed to provide an effective solution for solving the scattering problem in thick tissue imaging. The nonlinear optical effects govern the generation of the signals in a small focusing volume and provide a means to localize the signals. For example, in the most widely used variety—multiphoton fluorescence microscopy—molecules absorb simultaneously multiple photons in the near-IR to access the fluorescent states at energies that would require UV photons for direct absorption (9). The fluorescence induced by this nonlinear absorption process presents a nonlinear dependence on laser intensity—a quadratic dependence in the case of two-photon absorption (TPA). Thus fluorescence signal is generated only in the laser focal volume, which can be as small as a femtoliter. This assures that the signal collected by the detector is from the focal volume irrespective of how the emitted photons are scattered by the tissue. The images are then constructed by raster-scan of the focus spot in specimens on the focus plane. Three-dimensional imaging can be achieved by changing the focusing depth in specimens.

The same idea can be applied to detect many nonlinear optical effects that are well studied in the field of laser spectroscopy. They can be used as novel contrast mechanisms to provide molecular contrasts in biological imaging (10). Besides multiphoton-induced fluorescence, second harmonic generation, third harmonic generation (THG) and coherent anti-Stokes Raman (CARS) have been explored and used in tissue imaging (readers can find extensive discussion covering those topics in the recently published book *Handbook of Biomedical Nonlinear Optical Microscopy* [11]). Recently we have been focusing on exploring other

nonlinear optical contrasts (10) that are already well known in laser physics and spectroscopy, but not yet exploited extensively as an imaging contrast. Among them several nonlinear absorption-based contrasts have been successfully used in imaging several intrinsic molecules that lack fluorescence or emission in living tissues.

Absorption is linear if it is weak; the absorbed light power by a given sample is proportional to the incident irradiance; in other words, the absorption coefficient of the sample is independent of the incident light intensity. On the contrary, in nonlinear absorption the total amount of power absorbed by the sample has a nonlinear dependence with the incident light intensity; the absorption coefficient now depends on the incident irradiance. Many absorption mechanisms can cause nonlinear absorption. In TPA or multiphoton absorption (MPA), two photons or multiple photons excite molecules simultaneously in a single transition; the absorption increases quadratically for TPA or to the n th power for MPA with the incident intensity. Multiple photons can be also absorbed through stepwise processes—each photon is absorbed by the consecutive populated excited states; this process is essentially excited state absorption (ESA), and the excited states can be either singlet states or triplet states. The intensity dependence of ESA is actually more complicated than MPA; as shown later, it may have behaviors that mimic either MPA or saturation absorption (SA) depending on the absorption cross sections of each transition. Nonlinear absorption also happens when saturation effect occurs, where absorption decreases with increasing incident light intensity; this nonlinear behavior is due to the strong excitation process where stimulated emission depopulates the excited states and limits further absorption. The SA-like nonlinear absorption behavior can also be found when the depletion of the ground state population happens because of the existence of relatively long-lived metastates, which slow down the repopulation of the ground states and limit further absorption; this effect often refers as optical bleaching.

Nonlinear absorption processes mentioned above are clearly related to the electronic states of molecules, and measuring nonlinear absorption signals can provide molecular contrasts in imaging. Like any absorption measurement, the sensitivity depends on the dynamic range, which is determined (with the same detector) by the noise as the lower limit and the maximum laser intensity that can be used, as the upper limit. For the biological tissues the challenge is that the maximum laser intensity that can be used is always very modest in order to avoid damage. Nevertheless, conventional methods of measuring nonlinear absorption are usually not sensitive enough, or the way of measuring is not suitable for the imaging applications. For example, the most commonly used methodology for measuring nonlinear absorption is to measure the irradiance dependence of the absorbance by varying the laser intensity. During the course a plot of the transmission *versus* the irradiance can be generated and fitted to a specific theoretical model to extract the nonlinear absorption coefficients for the sample. The fitted model tells what type of nonlinear absorption effect possibly happens and the associated coefficients. The “open” aperture Z-scan method is one example in which such a plot is generated by moving a thin sample along a focusing beam where intensity varies with beam cross section. However, the way in which nonlinear absorption is determined by multiple measurements in these methods is not practical for imaging applications where a single measurement is generally required to yield at least one pixel of an image for the reason that the image acquisition time falls within a reasonable

range. For the past several years we have made quite an effort in developing the techniques that can measure nonlinear absorption sensitively and reasonably fast so as to fit imaging applications. The challenge of nonlinear absorption measurement as mentioned earlier is to identify a weak signal buried in a relative large background; the signal to background ratio may often be smaller than 10^{-4} . The strategy we have used to solve the problem is to measure such a small signal at a new frequency that is shifted out of the frequency band where the background resides. By using this “trick” we successfully extract the weak nonlinear absorption signal by using a single beam or double beams at different wavelengths (the pump-probe method). The single beam method can measure virtually any type of nonlinear absorption; however, so far only TPA or TPA-like ESA have been developed. In the single-beam method, one pulse train from a mode-locked laser is sinusoidally amplitude-modulated at one frequency; any loss due to linear effects, such as absorption and scattering, will stay at that frequency; however the nonlinear processes, such as TPA, will distort the sinusoidal modulation—a new frequency component will be generated due to the nonlinear processes. In the case of TPA, the new frequency will be presented at the second harmonic of the modulation frequency. So TPA can be measured by extracting the signal at that frequency. The double-beam method resembles the pump-probe transient absorption measurement: two pulses at different wavelengths act as the pump and the probe, respectively. If the pump pulse train is intensity-modulated at one frequency, the probe pulse train will catch that modulation if both pump and probe pulses are overlapped temporally and spatially. This method cannot only measure TPA and ESA but also differentiate them from the temporal behaviors when changing the interpulse delays—TPA has a symmetric cross-correlation trace according to the temporal overlapping point whereas ESA shows an instantaneous rise followed by decay due to the lifetime of the excited states. Besides optical bleaching and stimulated emission can be also measured in the experiments just as what we see in the transient absorption spectroscopy; those signals provide rich information about the photophysical properties of molecules, which no other method can offer, and can be used of course as imaging contrasts as well.

The methods we have developed provide the solution to the localization problem of nonlinear absorption signals and form the technical basis for nonlinear absorption microscopy. For helping understand the nonlinear absorption processes that are discussed here, this article will start with a brief survey of several important nonlinear absorption processes that may yield the signals in imaging by using kinetics models. We then summarize the principles of the methods that we have developed to measure TPA and ESA or transient absorption in general for imaging purposes. The realization of nonlinear absorption microscopy will be overviewed. This article will highlight several imaging applications we have been working on with some perspective in conclusion.

NONLINEAR ABSORPTION

Molecules absorb photons from the light field if the energy of photons matches that of a transition between the ground state and the excited states of the molecules. In general, the total number of photons absorbed per unit time N_{abs} is a function of the incident photon irradiance I , the absorption coefficient α and the illuminated sample volume V ,

$$N_{\text{abs}}(t) = \int_V d\mathbf{r} \alpha(\mathbf{r}, t) I(\mathbf{r}, t) \quad (1)$$

The linear absorption or nonlinear absorption depends on whether α is constant or dependent on the irradiance. The absorption coefficient α for the linear absorption is independent of the photon irradiance of the light field and found to be a product of the cross section of a particular transition and the total molecular density, *i.e.*

$$\alpha = \sigma(N_l - N_u) = \sigma N_{\text{total}} \quad (2)$$

where N_l and N_u are the number density in the lower and upper states of the transition, and N_{total} is the total number density of molecules in the light field and meets $N_{\text{total}} = N_l + N_u$. In linear absorption N_u is smaller enough to be neglected comparing with N_l .

The absorption coefficient for nonlinear absorption has a dependence on the irradiance. In this section we will go over several important nonlinear absorption processes to see how their signals depend on excitation intensity nonlinearly. Most of the absorption coefficients can be easily understood by solving the rate equations of kinetic models. Though the treatment is not as circumstantial as that in spectroscopy, it serves the purpose of understanding the basic physical processes and the data generated in nonlinear absorption microscopy.

Optical saturation

During a relative strong excitation, stimulated emission competes with absorption and results in light propagating in the same direction as the excitation light. The absorption decreases relatively when excitation intensity increases until a certain level at which no more light can be absorbed. This optical saturation effect can be easily understood by examining the rate equations of a two-level system in which two levels can be generalized from two electronic or vibrational levels with homogeneous and inhomogeneous broadening. The transition is simplified between two optically coupled states, a lower state and an upper state. The rate equations for a two-level system with nondegenerate states can be written as:

$$\begin{aligned} \frac{dN_l(t)}{dt} &= I_p \sigma (N_u(t) - N_l(t)) + k_{ul} N_u(t) \\ \frac{dN_u(t)}{dt} &= I_p \sigma (N_u(t) - N_l(t)) - k_{ul} N_u(t) \\ N_{\text{total}} &= N_u(t) + N_l(t) \end{aligned} \quad (3)$$

where $N_l(t)$ and $N_u(t)$ (m^{-3}) are the time-dependent number density populations in the lower and upper states of the transition, respectively. I_p (photons m^{-2}) is the excitation photon irradiance, σ the absorption cross section and k_{ul} the kinetic rate constant for the relaxation from the upper level to the lower level. The loss of the lower-level population is due to absorption, $-I_p \sigma N_l$, whereas the gain comes from stimulated emission, $I_p \sigma N_u$, and fluorescence or other mechanisms of depopulation of the upper level, $k_{ul} N_u$.

Solving the equations for the population difference under steady-state conditions, the absorption coefficient is:

$$\alpha = \sigma(N_1 - N_u) = \sigma N_{\text{total}} \frac{k_{ul}}{2I_p\sigma + k_{ul}} \quad (4)$$

As the photon irradiance is increased, the absorption coefficient decreases. If we define the saturation irradiance I_s , at which the absorption is decreased by a factor of 2, the absorption coefficient can be put in a convenient form:

$$\alpha = \sigma N_{\text{total}} \frac{1}{1 + I_p/I_s} \quad (5)$$

where $I_s = (2\sigma\tau)^{-1}$, and $\tau = 1/k_{ul}$

Optical bleaching

Optical bleaching is similar to optical saturation in which the absorption coefficient decreases with increasing excitation irradiance. The decrease in absorption is due to a decrease in the number densities of both the ground state and the state being excited. Ground- and excited-state depletion is normally caused by populating metastable states. Bleaching occurs when the returning rate from the metastable states to the ground state is slower than the rate of forming the metastable states. Photolysis can also cause optical bleaching due to a decrease in the number density of the absorbing species. Optical bleaching can be understood by using a simple three-level model depicted in Fig. 1. Levels 0 and 1 are the ground state and the excited state, respectively. The excited state relaxes reversibly only to level 2, which is a metastable state with a longer lifetime than that of the excited state. The rate equations for this system are:

$$\begin{aligned} \frac{dN_0(t)}{dt} &= -I\sigma_{01}N_0(t) + I\sigma_{10}N_1(t) + k_{20}N_2(t) \\ \frac{dN_1(t)}{dt} &= I\sigma_{01}N_0(t) - I\sigma_{10}N_1(t) - k_{12}N_1(t) + k_{21}N_2(t) \\ \frac{dN_2(t)}{dt} &= k_{12}N_1(t) - (k_{20} + k_{21})N_2(t) \\ N_{\text{total}} &= N_0(t) + N_1(t) + N_2(t) \end{aligned} \quad (6)$$

The N_i is the number density of absorbing species in level i , k_{ij} the rate constants for transitions from level i to j , σ_{ij} the absorption cross sections, I_p the excitation photon irradiance. After solving Eq. (6) by using $\sigma_{10} = \sigma_{01}$, the absorption coefficient is found to be

$$\alpha = \sigma_{01} N_{\text{total}} \frac{1}{1 + I_p/I_B} \quad (7)$$

where

$$I_B = \frac{1}{2\sigma_{01}\tau_{\text{effective}}}$$

and

$$\tau_{\text{effective}} = \frac{k_{12} + 2k_{20} + 2k_{21}}{2k_{12}k_{20}}.$$

Compared with Eq. (5) formulated for saturation, it is clear that optical bleaching exhibits irradiance-dependent behavior that is equivalent to that of optical saturation.

Multiphoton absorption

Two photons can be absorbed simultaneously by molecules in one optical transition as first described theoretically by Maria Goppert-Mayer (12,13) and experimentally demonstrated in the optical range 30 years later by Kaiser and Garret (14). This TPA can be understood as two photons undergo two transitions *via* virtual states that are between the ground state and the excited state. The TPA transition probability from the ground state, g , to an excited state, n , can be calculated by using the time-dependent semi-classical treatment of transitions (electrical field is not quantumized) and summing up that of all possible “virtual” intermediate states (15). The transition rate of TPA can be expressed in a convenient form in terms of a two-photon cross section,

$$R_{ng}^{(2)} = \sigma_{ng}^{(2)}(\omega) I^2, \quad (8)$$

with

$$\sigma_{ng}^{(2)}(\omega) = \frac{8\pi^3}{n^2 c^2} \left| \sum_m \frac{\mu_{nm} \mu_{mg}}{\hbar^2 (\omega_{mn} - \omega)} \right|^2 \rho_f(\omega_{ng} = 2\omega) \quad (9)$$

The total number of photons absorbed per unit time and unit volume in TPA is

$$\delta N_{\text{abs}} = N_{\text{total}} \sigma_{ng}^{(2)} I^2 \quad (10)$$

The absorption coefficient defined in Eq. (1) can be found as

$$\alpha = N_{\text{total}} \sigma_{ng}^{(2)} I \quad (11)$$

The transition rate of n -photon absorption can be found in a similar way (16), and the absorption coefficient becomes

$$\alpha = N_{\text{total}} \sigma_{ng}^{(n)} I^{n-1} \quad (12)$$

From Eqs. (10) and (11) we can see that the absorption coefficient for TPA is proportional to the photon irradiance of the incoming excitation, and the total loss of photons is proportional to the square of excitation irradiance. This nonlinear dependence is the foundation of the deep tissue imaging.

Excited state absorption and multiple-photon absorption

Similar to MPA, multiple photons can be consecutively absorbed by a molecule through its real intermediate states (compared with “virtual” states in MPA) (17). In the case of excited singlet or triplet intermediate states, multiple-photon absorption exhibits as ESA (reverse saturable absorption is also a commonly used term to describe the same effects [18]). The intermediate states can also be vibrational states, leading consecutive excitations to higher vibrational levels. As this type of absorption has completely different physics compared with MPA, “multiple-photon absorption” is used to differentiate it from MPA terminologically (19). Multiple-photon absorption can occur either through consecutive excitation of a species with double resonance (17) or *via* a stepwise process where the lower levels of the subsequent transitions are connected to the initially populated excited state by nonradiative relaxation routes (18). Excitation of the initially populated excited state will compete with excited-state relaxation. Unlike MPA, the absorbed photon density is first linearly dependent on excitation irradiance, but becomes nonlinear at higher excitation irradiances. The nonlinear irradiance dependence begins when the rate of secondary excitation of the initially excited state becomes competitive with the rate of returning to the ground state. The ESA can be modeled with a four-level system shown in Fig. 2 (19). The ground state, level 0, is excited by a one-photon process to the first excited state, level 1. This state reversibly relaxes to level 2, which may then either absorb a second photon or relax to the ground state. Separating levels 1 and 2 circumvents coherent effects in the multiple-photon absorption. The rate equations for this four-level system are

$$\begin{aligned}
 \frac{dN_0(t)}{dt} &= -(I_P\sigma_{01} + k_{02})N_0(t) + I_P\sigma_{10}N_1(t) + k_{20}N_2(t) \\
 \frac{dN_1(t)}{dt} &= I_P\sigma_{01}N_0(t) - (I_P\sigma_{10} + k_{12})N_1(t) + k_{21}N_2(t) \\
 \frac{dN_2(t)}{dt} &= k_{02}N_0(t) + k_{12}N_1(t) - (I_P\sigma_{23} + k_{20} + k_{21})N_2(t) + \\
 &\quad (I_P\sigma_{32} + k_{32})N_3(t) \\
 \frac{dN_3(t)}{dt} &= I_P\sigma_{23}N_2(t) - (I_P\sigma_{32} + k_{32})N_3(t) \\
 N_{\text{total}} &= N_0(t) + N_1(t) + N_2(t) + N_3(t)
 \end{aligned} \tag{13}$$

The absorption coefficient can be found out by solving the above equations under steady-state condition and calculating

$$\alpha = \sigma_{01}N_0 - \sigma_{10}N_1 + \sigma_{23}N_2 - \sigma_{32}N_3 \tag{14}$$

We can use several assumptions to simplify the steady-state solution of Eq. (13). The first assumption is that internal conversion of excited singlet or triplet states is rapid and stimulated emission rates are negligible, *i.e.* $\sigma_{10} = 0$ and $\sigma_{32} = 0$. Thus the absorption coefficient is

$$\alpha = \sigma_{01}N_0 + \sigma_{23}N_2 \tag{15}$$

The second assumption is the upward relaxation k_{02} is very slow and can be negligible, *i.e.* $k_{02} = 0$. The absorption coefficient can be calculated with Eq. (15) by solving the rate

equations in Eq. (13) under steady-state condition. If we drop the I_p^2 term, the calculation is much simplified and the absorption coefficient is

$$\alpha = \sigma_{01} N_{\text{total}} \frac{1 + I_p \sigma_{23} k_{20}^{-1}}{1 + I_p / I_S} \quad (16)$$

where

$$I_S = \frac{1}{2\sigma_{01}\tau_{\text{effective}}},$$

and

$$\tau_{\text{effective}} = \frac{k_{12} + 2k_{20} + 2k_{21}}{2k_{12}k_{20}}.$$

The absorption saturation happens when $I_p \sigma_{23} < k_2$, *i.e.* the relaxation rate of level 2 is much faster than the excitation rate from level 2 to level 3. The TPA kind of behavior (*i.e.* α is proportional to I_p) becomes prominent only when both $I_p < I_S$ and $I_p \sigma_{23} > k_{20}$ conditions meet. These relations imply that σ_{01} should be less than σ_{23} for TPA behavior to occur.

Pump-probe transient absorption

Thus far we have only considered the solution under steady-state conditions, which means the excitation is continuous and equilibrium is reached for all states. This would not be a problem if the time of populating excited states is shorter than the pulse duration and depopulation is slow enough. As short laser pulses are available in a broad tuning range, one can choose to prepare a specific state with a short laser pulse during which the population has no time to return to the ground state. This short pulse works as the pump to start the cascade of deexcitation processes while a weak pulse works as the probe to follow pathways and dynamics by interrogating the populations at various states. In the pump-probe experiment, the probe is chosen to measure the absorption coefficient changes between two optically connected states due to the pump process. The time-dependent data can be obtained by varying the arriving time of the probe pulse with respect to the pump. Tuning the probing wavelength or using broadband probing pulses, the measured absorption coefficient provides informative maps about the dynamics of metastable states or transient species. Interpretation of data has to be based on the model for a specific system. The model may include some or all of the following processes: ESA (from either singlet states or triplet states), stimulated emission (often from fluorescent states), optical bleach (often due to triplet states) and MPA (Fig. 3). The data from the experiments reflect the absorption difference with and without the pump presented, *i.e.* $\Delta\alpha'$. The probe beam basically measures the population differences between two states whose energy difference matches the probe photon energy. The ESA of singlet states and triplets have

$$\Delta\alpha' = \sigma_{23} N_2 \quad (17)$$

and

$$\Delta\alpha' = \sigma_{34}N_3 \quad (18)$$

respectively.

The stimulated emission and optical bleaching signals probe the same states

$$\Delta\alpha' = \sigma_{10}N_1 - \sigma_{01}N_0 \quad (19)$$

The absorption difference due to the pump is

$$\delta N_{\text{abs}}(t') = \int_V d\mathbf{r} \alpha(\mathbf{r}) I_{\text{pump}}(\mathbf{r}) \Delta\alpha'(\mathbf{r}, t) I_{\text{probe}}(\mathbf{r}) \quad (20)$$

where V is the overlapping volume of the pump and probe beam. The absorption of the pump results in the populations of excited states, which are time dependent with depopulation processes. As seen from the above equation, if α and α' are both independent of laser irradiance, the signal of the pump-probe experiment is proportional to the product of the irradiance of the pump and the probe pulses. The integral also determines the volume of sample giving out the signals, as a result of the spatial resolution in imaging.

PRINCIPLES AND IMPLEMENTATION OF NONLINEAR ABSORPTION MICROSCOPY

Methods for imaging nonlinear absorption

The most intuitive way to measure nonlinear absorption is probably varying the laser intensity to measure the irradiance dependence of the absorbance of incident light. The laser intensity can be adjusted by changing either the power or the beam size of the laser beam. The latter one is used in the Z-scan method, where a single, focused beam is used; and the sample is scanned along the propagating direction. An aperture is placed in the front of the detector to discriminate different nonlinear effects in samples by analyzing the transmitted power of the entire beam (open aperture) or part of it (closed an aperture) in the far field. In the open aperture Z-scan measurement the nonlinear absorption may yield a transmission attenuation behavior featuring a maximum at the beam waist and decreasing when the sample moves out of focus. This behavior is caused by the fact that the closer the beam waist, the higher the intensity. The amount of nonlinear absorption can be determined from the small transmission “dip” on a relatively large background. To make such a weak effect detectable, the peak intensities of laser pulses used in the experiment are usually very high and may reach the region where tissue damage occurs. For example, pulses from amplified systems were required in the recent TPA studies on Rhodamine 6G (20) and cytochrome *c* (21).

The methods that identify nonlinear absorption by varying the excitation intensity would not work in imaging applications, where a single measurement is expected to yield one pixel of an image at least. The secondary effects, such as fluorescence, induced by nonlinear absorption can be used for imaging nonlinear absorption. For example, multiphoton excited

fluorescence (22) or luminescence (23) provides an extremely sensitive way to measure MPA provided the emission of fluorophores is measurable. However, many molecules do not fluoresce; direct measurement of nonlinear absorption cannot be avoided. Recently several approaches based on frequency domain measurement have opened up windows for measuring nonlinear absorption without relying on the secondary effects or Z-scan methods (24,25). The essential idea is to place a spectral hole in the individual pulses by the ultrafast pulse shaping technique (26) and to detect the new frequency components inside the spectral hole, which are generated by the nonlinear absorption process. This method has been used in measuring TPA and SPM in imaging neural activation on mouse brain slices with the laser output at the level of 100 nJ per pulse (27). The low power version of this method has been also developed by using a mask to passively shape the pulses from a mode-locked laser system with 80 MHz repetition rate (25). For effective imaging applications, the imaging speed and sensitivity need further improvement.

In the above mentioned method single pulse spectrum is manufactured by pulse shaping techniques and new spectral frequency is created in the spectral hole by the nonlinear effects. The new frequency components can be also generated by nonlinear absorption if the pulse train is modulated. For example, amplitude modulation causes the intensity of each individual pulse to vary periodically. Any loss due to linear effects, such as absorption and scattering, do not change the intensity pattern in the pulse train; however, nonlinear processes, such as TPA, would reshape the intensity pattern and create new frequency components in the frequency domain. This approach allows us to measure nonlinear absorption fast and sensitively with a single laser beam (28). We can also use two laser pulse trains that overlap with each other spatially and temporally, with one of them intensity modulated; the modulation would transfer to the other beam if both beams are involved in the same nonlinear absorption process. If two pulse trains are at different wavelengths, they can be easily separated out by optical filtering. The pulse train shaping methods use laser pulses from mode-locked laser systems with high repetition rates, where the low noise floor makes the measurement very sensitive; thus relative low power pulses can be used in imaging. So the pulse train shaping methods with either one beam or double beams are the first choices for imaging biological samples. We will introduce their principles in detail in the following part of this section.

Single beam method—loss modulation measurement

As shown in Fig. 4, consider a high-stability pulse train from mode-locked laser with repetition rate f_0 and a power spectrum with components at nf_0 ($n = 0, 1, 2 \dots$). A relatively slow sinusoidal amplitude modulation at a frequency f makes new frequency components appear at one upper and one lower sideband of nf_0 with the offset of f . The amount of absorption varies among pulses with different intensity because of nonlinear absorption; as a result, the perfect sinusoidal amplitude modulation is distorted. In the case of TPA, the distortion results in extra sidebands created at $\pm 2f$ which would not be generated by linear processes. Measuring the $2f$ component (the side band generated at the CW component) allows the extraction of the small TPA signal buried in a large transmitted power. In the experiment, the sinusoidal modulation is generated by a Mach-Zender interferometer, in which two arms are frequency shifted with a frequency difference f . The modulated laser

beam is then focused in the sample. The transmitted light is detected by photodiodes and different frequency components ($2f$ and $1f$) are extracted by a lock-in amplifier. If the thickness of the sample is much larger than the Rayleigh range of the laser beam, for TPA media, the ratio of the amplitude of the signals measured at $2f$ ($2f$ component of TPA signal) and $1f$ ($1f$ component of transmitted light) can be calculated as (28):

$$\frac{P_{2f}}{P_{1f}} = \frac{0.66nC\delta}{\tau\lambda f_0} \arctan\left(\frac{L}{2nZ_0}\right) P_{1f} \quad (21)$$

where δ , C and n are the TPA cross section, the molecular density and the refractive index of the sample, respectively; τ and λ are the width and center wavelength of the pulse, respectively; and L and Z_0 are the total path length of the sample and the Rayleigh range of the incident beam in air ($Z_0 = \pi r^2/\lambda$, where r is the radius of the focal point), respectively. In experiments, P_{2f}/P_{1f} can be calculated from the readings of the lock-in amplifier, while P_{1f} should be the amplitude of the sinusoidal modulation.

The sensitivity of this method is determined by the spurious signal appearing at $2f$. One major source comes from the nonlinear optoelectric response of photodetectors and related circuits. This type of signal appears as a background and varies with the light power exposed to the detector sensitive area. To minimize the nonlinear effect of photodetectors, the light intensity should be controlled under certain levels (detector dependent), and this limits the maximum intensity used in the measurement. This method can routinely measure 10^{-5} absorption changes with pulse energies of only a few pico-joules. The sensitivity of this loss modulation method is ultimately limited by shot noise. The TPA cross section of Rhodamin 6G has been successfully measured by this loss modulation method (28).

Double beam method—pump-probe transient absorption measurement

The double beam method for measuring nonlinear absorption resembles the traditional pump-probe transient absorption experiments. The two beams may both come from the same laser source or from two different laser sources but synchronize with each other, so the two pulse trains, even at different wavelengths, have well-defined and controlled timing. As shown in Fig. 5, one of the pulse trains acts as the pump, which excites molecules to populate the excited states initially; the other pulse train acts as the probe, which, as discussed earlier, measures the absorption change due to the pump. By continuously changing the timing between the pump and the probe pulses with an optical delay line, the dynamic change in populations in each state can be monitored. In this approach the pump is modulated at a certain frequency, resulting in modulated excited state populations; if the probe is tuned to the right wavelength, the transmission of the probe will “catch” that modulation frequency as the transient absorption signals. This approach has been widely applied to investigating transient species in photochemistry and photobiology, whereas most of the experiments are performed on amplified systems. It has been realized earlier (29) that the noise in the pump-probe experiment can be reduced by using high-repetition rate mode-locked laser pulse trains and high modulation frequency, due to less noise from laser and modulation system at higher frequency. The mode-locked laser systems have proved to be the best ultrafast laser sources for imaging applications. One argument about the high repetition rate is that during the period between two excitations the processes measured may

not be completely finished, and thus instead of the dynamics of the processes themselves accumulation effects are measured. It would be a serious problem especially when heating effects are involved; not enough time to dissipate heat may cause thermal damage of tissues. An adjustable repetition rate may help mitigate the thermal effect.

The signals in the pump-probe experiments are ultimately measured by lock-in amplifiers. A lock-in amplifier has the capability of performing phase-sensitive detection (PSD), which requires an input of frequency reference. In practice we can set the same phase as the measured signals to achieve maximum reading in the lock-in amplifier. We can also differentiate the different types of signals from the phase information. For example, if we set the same phase with the absorption signals (loss), the bleaching and stimulated emission signals appear at the phase with 180° difference, or negative signals (gain). A dual phase lock-in amplifier, such as Stanford Research SR830, has two PSDs with a fixed 90° phase difference in reference. The outputs of two PSDs eliminate the phase information of the signals; on the other hand, the phase between the signal and lock-in reference can be calculated by

$$\phi = \tan^{-1}(Y/X) \quad (22)$$

where ϕ , Y and X are the phase, the quadrature output and “in-phase” output of the lock-in amplifier, respectively. If we set the “in-phase” channel X to the same phase with the pump signal, the phase we obtain from the lock-in amplifier is the phase of the pump-probe signal

$$\tan(\phi) = 2\pi f\tau \quad (23)$$

where f is the modulation frequency and τ is the lifetime of the process.

In most ultrafast spectroscopy studies, one is interested in the processes with lifetimes that are shorter than 1 ns; for the lifetime of 1 ns, with a modulation frequency of 1 MHz, we can calculate that ϕ is $1.1 \times 10^{-4}^\circ$, which is too small to be noticed. However, if the lifetime of the signal is increased to 1 μ s, still with 1 MHz modulation frequency, ϕ is 80° and will have a large effect in the PSD like the lock-in amplifier. Long-lived processes complicate the phase measurement and appear as a constant “background” in the lock-in signals. Similar to the frequency domain method for measuring the fluorescence lifetime, the lifetimes of long-lived processes can be obtained from the phase measurement when varying the modulation frequency. Using this method we found 0.148 and 0.293 μ s species in red hair melanosomes and black hair melanosomes, respectively (30,31).

As shown above, the pump-probe transient absorption measurement provides several types of nonlinear absorption signals that can be used as imaging contrasts. TPA appears as an instantaneous absorption signal rising only within the temporal overlap of the pump and the probe pulses. ESA appears as an absorption signal with an instantaneous rise followed by a decay according to the depopulation processes of excited states. Stimulated emission and optical bleaching appear as gain signals with dynamics similar to that of ESA signals. The phase measurement can not only differentiate among types of nonlinear absorption but also reveal long-lived species that have lifetimes exceeding the period of pulse repetition. Compared with the loss modulation method, the pump-probe method is less affected by the

nonlinearity of optoelectronic circuits, and thus the dynamic range is improved by 1 order of magnitude.

Implementation of nonlinear absorption microscopy

The nonlinear absorption microscopy can be realized on a non-descanned multiphoton fluorescence microscope with some modifications for adapting the nonlinear absorption measurement. There have been plenty of publications (32–34) and internet resources about constructing a single-point-scanning two-photon fluorescence microscope. Here we present a typical microscopic setup that has been used in our nonlinear absorption imaging experiments and review some of the key points that need to be considered for performing nonlinear absorption microscopy.

Figure 6 shows a schematic diagram of a typical system used in our imaging experiments, which consists of an ultrafast laser system, a laser intensity modulation system and a laser scanning microscope. The ultrafast laser system includes a femtosecond mode-locked Ti:sapphire laser (Tsunami, 80 MHz, 100 fs; Spectra-Physics, Mountain View, CA) tunable from 690 to 1080 nm and a synchronously pumped optical parametric oscillator (Opal; Spectra-Physics) extending the output wavelength to 1300–1600 nm. The outputs of two lasers provide at least two synchronized pulse trains at different wavelengths that can be used in two-color pump-probe experiments. For the single beam TPA measurement, a Mach-Zender interferometer is required to generate the sinusoidal modulation with minimum background from the higher harmonics. For the pump-probe experiments, two laser beams at different wavelengths are generally used, and an acousto-optic modulator is good enough for intensity modulation. The microscope in upright configuration is assembled on an XT95 rail (Thorlabs), on which were attached in succession an XY 2-axis scanning mirror (6210; Cambridge Technologies), a scan lens (Linos), a trinocular (Zeiss) with a tube lens and a focusing unit (Nikon). The scanning mirror, the scanning lens and the tube lens are aligned so that the pivot point on the scanning mirror is imaged on the back aperture of the objective. As the incoming laser beam is directed by the scanning mirror in the *X* and *Y* directions (the one direction is much slower than the other one), raster scans of the focus spot are formed on the focus plane of the objective. For two-photon fluorescence imaging, the fluorescence generated at focus spots is collected by the imaging objective and reflected by a dichroic mirror (670DCXXR; Chroma) onto a photomultiplier tube (PMT, HC120–15; Hamamatsu). IR blocking filters (BG39; Schott Glass) are placed before the PMT to cut off the excitation wavelength. The voltage signal from the PMT is then digitized by a multifunction data acquisition board (PXI-6115; National Instrument) in a PXI chassis (PXI-1042; National Instrument). The analog outputs of the PXI-6115 also provide the voltage patterns to drive the scanning mirrors. Either a modified Matlab (Mathworks) program based on ScanImage (35) or a homebuilt program written in Labwindows/CVI (National Instruments) is used to run the scanning microscope and acquire images. The samples are held on a simple microscope platform attached to a three-axis motorized translation stage (assembled by two MFN25CC and one UZM80PP; Newport) driven by a universal motion controller (ESP7000; Newport). The UZM80PP is a vertical translation stage with a minimum incremental motion of 0.1 μm and moves the sample up and down in the *Z*-direction. Combination of the *XY*

scanning and the Z translation movement scans the focus spot through volumes of samples and realizes the three-dimensional imaging.

In the single-beam imaging experiments, nonlinear absorption images are usually acquired in the transmission mode and at the same time the two-photon fluorescence (TPF) images can be acquired in epi-mode (back-reflection mode). In this case the sinusoidally amplitude-modulated laser pulses from the interferometer are sent into the microscope and focused by an objective onto samples on the stage. The PMT output is sent to a lock-in amplifier (SR844; Stanford Research) for extracting the fluorescence signal at the modulation frequency ($1f$, as shown in the section about the method). The signal to noise ratio is improved by this narrow band amplification and detection. The laser beam transmitted through the specimen is collected by a condenser and detected by an amplified photodiode (PDA 55; Thorlabs). The signal from the photodiode is sent to another lock-in amplifier (SR830 or SR844; Stanford Research) for extracting the $2f$ components, where the TPA signal is presented. The analog outputs of both lock-in amplifiers are digitized by a multifunction data acquisition board PXI-6115. In this case one of the A/D channels is used for the fluorescence signal, while the other two A/D channels have to be used to collect the TPA signals from both channels (denoted as X and V) of the dual phase lock-in amplifier as the TPA signals might show up in both channels if the phase of signal cannot be determined. Note that the analog outputs from the lock-in amplifier are used to avoid the complication associated with digital data transfer *via* the GPIB port. The images are then constructed in the same way as the fluorescence mode, but with additional channels for TPA images. The image acquisition speed is limited solely by the lock-in detection because the pixel dwelling time has to be at least twice as long as the time constant. Typically the scanning speed is set at 100 ms per line, in result, about 52 s for acquiring one frame with 512×512 pixels.

For two-color transient absorption imaging, if images are collected in the transmission mode, the microscope setup needs only a slight modification to accommodate the two collinear laser beams entering the microscope; proper filters are required to cut off the pump wavelength. The lock-in amplifier now detects the signal at the same frequency as that of the modulation. The nonlinear absorption images could be also acquired in back-reflection mode as in the fluorescence imaging. In the experiment, the probe light back-scattered after the focus is expected to carry the frequency component induced by the pump beam, though it has been scattered many times before reaching the detector. Unlike fluorescence, the signal buried in the back-scattered light has the same wavelength as the probe beam; the dichroic mirror has to be replaced by a polarizing beam splitter, which separates the incoming beam and back-scattered beam by their polarizations. We set the incident pump and the probe beam to p polarization so that they can pass the polarizing beam splitter completely. The backscattered light is very likely depolarized and its s polarization component can then be reflected by the beam splitter. An avalanche photodiode (APD; Hamamatsu) is used for signal detection and provides the appropriate sensitivity which lies between PMTs and amplified PDs.

IMAGING APPLICATIONS OF NONLINEAR ABSORPTION MICROSCOPY

Imaging cutaneous pigmentation

Pigmentation of the skin is mainly determined by the amount of cutaneous melanin, which is synthesized within the subcellular organelles of melanocytes, melanosomes, and distributed through dendritic extensions of melanocytes to surrounding keratinocytes. Two types of melanin are generally found in skin: eumelanin, a black-to-dark-brown insoluble material, and pheomelanin, a yellow-to-reddish-brown alkali-soluble material (35,36). Although extensively studied, neither the biological functions nor chemical structures of melanin have come to conclusions yet (36,37). The primary function of cutaneous melanin is generally believed to be photoprotection (for a recent review, see Brenner and Hearing [38]). It is supported by the observation of “supranuclear melanin caps” (39,40) located over nuclei of keratinocytes and thought to act as “shades” to block the harmful UV radiation. It is further supported by melanin’s scavenging capability (41) of reactive oxygen species generated by sun light and extensive epidemiological data that have revealed an inverse correlation between skin pigmentation and the incidence of sun-induced skin cancers (42–44). Considering melanosome’s high mobility (45) and sensitive response to light (46–48), it is important to monitor their distributions in skin in studying melanin metabolism functions and, moreover, histopathology roles in clinical applications. For example, the morphology and location of melanin-containing cells, melanocytes, are often found to be atypical in the diseases related to melanocytic dysfunction, and migration of melanocytes is often found to be a sign of malignancy in melanoma (49). The morphological features indicated by the melanocyte nesting as well as cell shape and cell size provide important diagnostic differentiation between benign and malignant melanocytic skin lesions (50–52).

Melanin is not an efficient emitter (quantum yield $< 10^{-3}$) but a good quencher due to its broad absorption band. However, melanin granules have been suggested to contribute a considerable amount of fluorescence from skin cells in fluorescence-based imaging methods, such as TPF microscopy. Along with other intrinsic molecules, such as reduced nicotinamide adenine dinucleotide, flavins, elastin, collagen, keratin and porphyrin, melanin or melanin granules provide the endogenous fluorescence contrast for imaging skin cells and dermal structures (53,54). The reflectance confocal laser scanning microscopy, a scattering-based imaging modality, provides the strong contrast of melanin granules because of their significantly higher refractive index ($n = 1.7$) than that of the surrounding cytoplasm ($n = 1.35$). However, the comparison *in vivo* imaging studies (55) performed by a combined TPF and reflectance confocal laser scanning microscope on the normal skin located at the back of the hand did not show matching patterns of melanin distribution; additionally, the fluorescence images did not present any distinct difference between melanin granules and surrounding cytoplasm. This raises a question about the fluorescence source in melanin granules: whether the observed fluorescence comes from melanin or other intrinsic molecules within granules. It is difficult to answer this question by the fluorescence spectroscopy study for almost all intrinsic fluorophores are heavily overlapped with each other spectrally in fluorescence emission and have even no significant differences in fluorescence lifetimes (53). However, the argument from the reflectance confocal studies is not strong enough because the refractive index variation cannot provide the reliable

molecular contrast (as such, optical coherent tomography seeks additional mechanisms to enhance the molecular specificity in imaging [56–59]).

So we need to seek for other contrast mechanisms that originate from melanin itself. Melanin has a well-known broad featureless absorption spectrum extending to near-IR. Recent studies have suggested that sequential multiple photon absorption may occur in melanin and allow efficient multiple photon excitation with near-IR light (60,61). We have confirmed that melanin has TPA-like ESA by using both one-wavelength loss-modulation and two-color pump-probe methods (62,63). The depopulation of the excited states is characterized as a nonexponential decay fitted to a double-exponential model with lifetimes of 450 ± 50 fs and 3.0 ± 0.5 ps. It turns out that the ESA signal has provided excellent contrast for imaging melanin as demonstrated by both the single-wavelength method and the two-color transient absorption method in both *ex vivo* and *in vivo* experiments.

In single-wavelength nonlinear absorption imaging as described earlier, the ESA of melanin provides the similar contrast mechanism as TPA. Excellent contrasts from the ESA of melanin were first demonstrated in imaging-embedded B16 cells, a type of mouse melanoma cells that produce melanin, in *ex vivo* and *in vivo* mouse models. The power of this technique has further been demonstrated in imaging melanoma cells in a fixed human melanoma lesion. In that experiment, the laser was tuned to 780 nm and intensity-modulated at 25 kHz. Both two-photon-induced fluorescence and the two-photon absorption images were acquired simultaneously. A pair of such images is shown in Fig. 7a,b, respectively. The ESA image (Fig. 7a) shows mainly the distribution of melanin in the skin layer. In the TPF image the autofluorescence signal from the intrinsic molecules indicates the cell distribution in different skin layers; as the nuclei of cells appear dark and rounded, the epidermis is differentiated by its higher cell density from the dermis. Although the TPF image provides a lot of useful information about cell organization in skin layers, the pigmentation information is missing. However, as shown in Fig. 7c, if we superpose two images to each other, the combined images provide an astonishing picture about how the pigment is distributed among cells and skin layers. The dendritic melanin structure can be clearly seen at the epidermis-dermis junction and extending to the epidermis layer. These dendrites are considered as an indication of abnormal development of the melanocytes. In the z-stack imaging mode, a volume of tissue can be scanned and a stack of images can be acquired at different focusing depths. Image processing software can help visualize the data by using 3D display algorithms. For example, the image in Fig. 7e is constructed by using 3D volume rendering algorithms from a stack of 22 ESA imaging slices with a depth increment of $5 \mu\text{m}$ on the same lesion. Such a 3D image provides a way to evaluate the continuous pigment network in the skin lesion, and it would not be possible for histopathology to do that without destructing the whole lesion.

In the two-color transient absorption imaging the dynamic range can be improved about 1 order of magnitude than the single-wavelength ESA imaging (62,64). Together with the improved sensitivity by using an APD the epi-mode ESA imaging was first demonstrated on hair samples (31). In principle, any probe light scattered backward after the focus carries the same modulation frequency as the transmission light does and ultimately reaches the detector after scattering multiple times. Here scattering is somewhat helpful for collecting

more signals. The success of epi-mode imaging suggests that this imaging technique can be potentially applied to *in vivo* imaging. High quality ESA images have been also acquired in imaging melanin granule distribution on slides of fixed human melanoma sections. Figure 8 presents one of the slide images acquired with the 810 nm pump and the 740 nm probe. Figure 8a shows a bright field image by stitching a set of 10 photographs taken from a thin tissue section of invasive skin melanoma lesion. Without staining, the melanin granules can be barely observed in the bright-field image, mostly along the basal layer as yellow to brown spots. Figure 8b is the ESA images acquired from the same area as shown in Figure 8a. Skin layers can be easily recognized: the stratum corneum is seen with a bright line due to strong signal from the blue ink mark (left over from tissue sectioning procedure), and the basal cell layer is identified as a continuous ridge with a strong melanin signal. The normal basal cell layer appears as a clear thin wall that separates the epidermis from the dermis. Growth of melanoma causes severe disruption of the integrity of the ridge pattern at the basal layer. In the ESA image, the invasive melanoma onset can be found with the features of disconnected melanin distribution, unclear ridge shape, melanocyte infiltration to both the epidermis and the dermis and large variation of cell shape and size. The high resolution images (Fig. 8c,d) enlarge the two selected areas (marked with boxes in Fig. 8b for a better view).

Nonlinear absorption microscopy imaging of red blood cells

Imaging vascular structure, blood flow dynamics and blood oxygen level in microscopic level provide crucial information in studies, such as angiogenesis of normal and cancerous tissues and hemodynamics related to cerebral function. By using the contrast agents or fluorescent dye, blood plasma and vessel walls can be labeled and imaged by fluorescence-based microscopy (confocal detection or two-photon excitation). These methods can measure a number of important parameters such as blood flow rate, blood vessel diameter, vascular density and endothelial permeability. However, one important parameter is often missing—oxygen saturation level. To image oxygen saturation level, red blood cells (RBCs) have to be targeted for its role as oxygen transporter by binding oxygen to hemoglobin. Oxygen-bonded hemoglobin (oxyhemoglobin, oxyHb) and oxygen-dissociated hemoglobin (deoxyhemoglobin, deoxyHb) have distinct absorption spectra in a broad range. So one straightforward method is to measure the absorption spectra pixel by pixel during blood imaging, and the absorbance measured at two wavelengths should allow us to calculate the concentrations of oxyHb and deoxyHb at each pixel. This hyper-spectral imaging method has provided very good oxygen level images within a thin layer of tissues (65,66). As discussed earlier, this type of linear absorption imaging would not be able to image thick tissues; so a number of nonlinear optical signals of hemoglobin, such as nonlinear absorption (66–68), CARS (69) and THG (70,71), have been explored in the hope of finding nonlinear signatures for imaging Hb with oxygenation state. Up to date only the nonlinear absorption method (66,68) has been successfully demonstrated in imaging RBCs *in vivo*. In the nonlinear absorption imaging experiments two-color pump-probe absorption method was used to measure the ESA of hemoglobin.

The two-color pump-probe spectroscopy studies in cuvette samples was first performed to investigate the signals and dynamics of the ESA in Hbs. A dramatic difference in signal sizes was found between oxyHb and deoxyHb when choosing certain combinations of the

pump and the probe wavelengths. As shown in Fig. 11a, the maximum difference has been found so far by using 740 and 810 nm pulses as the pump and the probe wavelengths, respectively. However, both hemoglobins show similar dynamics of the excited state depopulation.

The ESA signals of Hb provide intrinsic contrasts in imaging; in other words, no dye labeling or any exogenous molecules are introduced to cells or tissues for imaging. The first image (Fig. 9b) of RBCs was acquired from fixed cells on a slide with the pump at 775 nm and the probe at 650 nm (66). The laser power levels—3.3 mW for the pump (775 nm) and 1.5 mW for the probe (650 nm)—were quite comparable with those used in the TPF imaging (72,73). The interpulse delay is fixed at 100 fs where the maximum signal presents. The three-dimensional image shown in Fig. 9c was constructed by volume rendering from a stack of 10 images acquired in Z-direction with 1 μm depth increment. The featured biconcave shapes of RBCs can be clearly observed. Under the same imaging settings, blood vessels in the ear of a black mouse were imaged right after the mouse was killed. Figure 10 shows a bright field image and a series of images taken at various depths from 20 μm down to 70 μm below the skin surface. The strong signals coming from the epidermis layer (0–40 μm) (66), are mostly from the melanin in hair follicles of the skin with some possible contributions from other skin constituents such as collagen. At the level of the dermis (40–70 μm deep in the tissue), the images reveal hundreds of individual blood cells lining inside the vessels. Following streams of RBCs one can appreciate the complex architecture of the vasculature in skin tissues. Note that the RBCs observed in those images are almost static because the mouse was killed and the normal blood flow was stopped. In the live animal, RBCs can move at a speed up to a few millimeters per second; thus the dwelling time of each pixel decreases and the imaging S/N reduces. Under improved conditions of imaging, *in vivo* imaging of microvasculature was demonstrated on imaging ear microvascular structures in an anesthetized nude mouse (CD1; Charles River Laboratory) (68). The improvement included several aspects: the pump and the probe wavelengths were tuned to 735 and 810 nm, respectively; a low power (20 $\times$) objective (Olympus PlanApo) with high numerical aperture (0.7) was used to increase the field of view while still keeping the focus spot small; the interpulse delay was set at 500 fs to minimize the unwanted signals from cross-phase modulation or sum-frequency generation of the skin tissue. Micro-vessels in the range of 5–10 μm were still clearly visible at the depth of about 70 μm under the skin surface with low laser power levels. Such an image acquired at 30 μm is shown in Fig. 11c. By switching the pump and the probe wavelengths, *i.e.* 810 nm for the pump and 735 nm for the probe, the image acquired in the same region at the same depth is shown in Fig. 11d. Comparing images in Fig. 11c,d, stronger overall signals are found in Fig. 11d possibly due to higher power of the pump and lower loss of the probe (810 nm) in transmission. The most important feature brought by switching the pump and the probe wavelengths is the relative change in the signals: some vessels, as indicated by blue arrows, have much more prominent change than the vessels indicated by a red arrow. As shown in the cuvette samples, oxyHb and deoxyHb do show different ESA signals when using the different combination of the pump and probe wavelength: this difference implicates that the blue-arrowed vessels in Fig. 11d are venules, and the red-arrowed vessels are arterioles. These preliminary data are

encouraging and suggest that the two-color ESA imaging can potentially be used for *in vivo* imaging of the oxygen levels in the vessels. Further quantitative studies are awaited.

PERSPECTIVES

Nonlinear absorption is one of the fundamental processes in light-matter interaction. As a powerful spectroscopy tool nonlinear absorption measurement has provided important information about atomic and molecular structure and dynamics, which are also sensitive to the local environment (74). By utilizing the recently developed imaging platform, we have demonstrated that nonlinear absorption can be used as a molecular contrast mechanism for imaging melanin and hemoglobin in tissues. The significance of nonlinear absorption imaging is that the signal is “original,” no labeling and expressing needed: the signal is transient, allowing observation of fast photo-activated species; further, the signal provides more spectroscopic information than fluorescence (mostly from the first excited states [75]). Of course the sensitivity of nonlinear absorption measurement is not as good as that of fluorescence measurement. Fortunately both melanin and hemoglobin have considerably high concentrations in their physiological existence—melanin in melanosomes and hemoglobin in RBCs. So searching for the next molecular targets should focus on those molecules that tend to aggregate to each other. The image acquisition speed of the current nonlinear absorption microscope is mainly limited by lock-in detection. The recent development of multifocal scanning techniques (76–78) and smart detector arrays (79–81) may possibly help overcome the bottleneck of imaging speed.

In the two-color pump-probe transient absorption imaging we have demonstrated that we can image oxyHb and deoxyHb separately by using different combinations of the pump-probe wavelengths. This is based on their differences in the wavelength-dependent ESA strength. Previous studies have shown that two types of melanin, eumelanin and pheomelanin, present significant differences in their excited-state spectra upon UV excitation (82). It suggests possibility of imaging the distribution of eumelanin and pheomelanin separately. Characterizing eumelanin and pheomelanin in skin tissues is of great interest for a number of reasons. It may provide tools to study their possibly contradictory roles (83,84) in photoprotection under native conditions. As suggested by the high susceptibility to skin cancers among fair-skinned individuals from epidemiological studies (84–86) and increased photoreactivity of pheomelanin in the UV light (87–89), the ratio between the contents of two types of melanin may be used as a quantitative measure for skin cancer susceptibility. The imaging technique that allows examining different melanins *in vivo* will greatly benefit those researches without going through the destructive procedures of chemical analysis. Moreover, the study of concentrations of two melanins in melanoma lesions (90,91) suggested that the composition ratio may have clinical value for diagnosis (64). Therefore, the differentiation of eumelanin and pheomelanin in skin tissue can provide information about changes in melanogenesis and potentially be useful for clinical evaluation of the progression stage of melanoma. The early attempt of using ESA signals alone has shown very little difference between eumelanin and pheomelanin (64); however, Fu *et al.* (31) have shown that the ratio between the ESA and the long-lived signals is significantly different in red hair and black hair melanosome samples, and suggest that the

ratio imaging mentioned above may be a practical approach, though further demonstration in skin tissues will have to be made in future studies.

It is worth mentioning that the pump-probe microscopy (92,93) was first realized by Cheng-Yuan Dong in Enrico Gratton's laboratory in the frequency-domain by detecting stimulated emission. The key idea of their technical approach was to utilize the slightly different repetition rates between the pump and the probe pulse trains to yield incremental interpulse delays automatically and periodically. The dynamics of the pump-probe signal was then sampled with a temporal interval that equaled the period difference of two pulse trains (94,95). The advantages of this method are: no intensity modulation is needed; the full dynamics can be constructed very fast due to elimination of the optical delay line, which is usually driven by slow mechanical translation stage. The drawback is that temporal studies require a digital oscilloscope, whose noise suppression is not as good as a lock-in amplifier. We follow the traditional way of acquiring transient absorption signals by using an optical delay line; the interpulse delay is fixed during image acquisition. However, in some imaging applications, if we do not consider the temporal behavior of the signal, the above mentioned asynchronous sampling method may help increase the signal size.

Nonlinear absorption microscopy is primarily developed for increasing molecular contrast mechanisms in imaging molecule constituents in tissues. Such a microscope can also be used as a platform for doing spectroscopy in a small volume defined by diffraction limited focusing, and offers the means to investigate the molecular dynamics in a microenvironment that is confined to cell membranes or to subcellular compartments. It is interesting to see how different it is compared to that in a cuvette solution.

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Figure 1.
Three-level energy diagram used to describe kinetic effects of optical bleaching.

Figure 2.

A kinetic model for excited state absorption. SE = stimulated emission; Abs = absorption.

Figure 3.

The processes that can yield signals in the pump-probe transient absorption measurement. 2PA = two-photon absorption; 1PA = one-photon absorption; OB = optical bleach; SE = stimulated emission; ESA = excited state absorption.

Figure 4.

The principle of single beam two-photon absorption measurement. A sinusoidal intensity modulation (10 MHz) of the pulse train from a mode-locked laser (80 MHz) generates sidebands (10 MHz away from 80 MHz and its harmonics) in the frequency domain. Two-photon absorption creates new sidebands in the transmitted light. The new sidebands shift away from the repetition frequency with double the frequency (20 MHz) of the modulation.

Figure 5.

The principle of the pump-probe transient absorption measurement. The change in transmission (ΔT) of the probe, due to the pump process, reflects the population changes of the ground state and the excited states. The time-evolution of signals can be drawn by varying the interpulse delay between the pump and the probe pulses.

Figure 6. Schematic for a typical laser-scanning microscope setup for performing nonlinear absorption microscopy.

Figure 7.

Imaging a fixed human melanoma lesion with a single laser beam. Image size: $266\ \mu\text{m} \times 266\ \mu\text{m}$ (a) TPA image taken at $20\ \mu\text{m}$ deep in the lesion, (b) TPF image taken at $20\ \mu\text{m}$ deep in the lesion. The nuclei of cells appear in dark round shape due to lack of fluorophores in nuclei, (c) The superposition of the TPA (in gray scale) and TPF (colored) images clearly shows melanin distribution in the skin layers, (d) Bright field image. The dark circular patterns show the epidermis–dermis junction layer containing the malignant melanocytes. (e) 3D volume rendering of TPA signal from human melanoma lesions, which was constructed from 22 imaging sections. The size of the block is $266\ \mu\text{m} \times 266\ \mu\text{m} \times 105\ \mu\text{m}$.

Figure 8. Mosaic of five images of an invasive melanoma skin slice in the (a) bright field images of an invasive skin slice taken with a DCM 130 camera; (b) mosaic of 10 laser scanning images of the same skin slice acquired with two-color transient absorption microscopy; (c, d) higher magnification two-color transient absorption images taken with a 40× objective at the two locations indicated with the orange boxes and arrows at the same power levels. Adapted with permission from Fu *et al.* (31).

Figure 9. (a) Bright field imaging and (b) laser scanning two-color TPA image of mouse RBCs. (c) The reconstructed 3D cell image is based on 10 layers with layer separation of 1 μm and image size of $40\ \mu\text{m} \times 40\ \mu\text{m} \times 10\ \mu\text{m}$. Adapted with permission from Fu *et al.* (66).

Figure 10. Bright field image and a series of laser scanning two-color ESA images at various depths in the black mouse ear. The pump is at 650 nm (2.4 mW), and the probe is at 775 nm (1.4 mW). Adapted with permission from Fu *et al.* (66).

Figure 11.

Comparison of the ESA signals of oxyhemoglobin and deoxyhemoglobin with different pump-probe combinations. Arterioles and venules can be discerned in vessel images, (a) ESA signals with 810 nm pump and 735 nm probe; (b) ESA signals with 735 nm pump and 810 nm probe; (c) blood vessel imaging with 810 nm pump and 735 nm probe; (d) blood vessel imaging with 735 nm pump and 810 nm probe. Adapted with permission from Fu *et al.* (68).