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REVIEW

Pump-probe optical microscopy for imaging nonfluorescent chromophores

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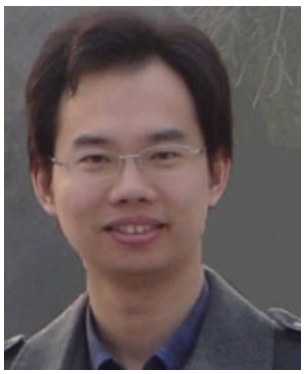
Abstract Many chromophores absorb light intensely but have undetectable fluorescence. Hence microscopy techniques other than fluorescence are highly desirable for imaging these chromophores inside live cells, tissues, and organisms. The recently developed pump-probe optical microscopy techniques provide fluorescence-free contrast mechanisms by employing several fundamental light–molecule interactions including excited state absorption, stimulated emission, ground state depletion, and the photothermal effect. By using the pump pulse to excite molecules and the subsequent probe pulse to interrogate the created transient states on a laser scanning microscope, pump-probe microscopy offers imaging capability with high sensitivity and specificity toward nonfluorescent chromophores. Single-molecule sensitivity has even been demonstrated. Here we review and summarize the underlying principles of this emerging class of molecular imaging techniques.

Keywords Pump-probe microscopy · Label-free imaging · Excited state absorption · Stimulated emission · Ground state depletion · Nonfluorescent chromophore

Introduction

Light microscopy has become indispensable for studying live cells and organisms. The ability to image specific biological molecules and to follow their spatial distribution and temporal dynamics has become essential for understanding their physiological roles and regulatory mechanisms. Advances in optical imaging techniques have significantly expanded our knowledge of biological processes on the microscopic scale. Fluorescence is currently the most popular optical contrast mechanism, thanks to its unprecedented sensitivity of background-free detection [1, 2]. The power of fluorescence microscopy is further enhanced by exquisite labeling specificity with genetically encoded fluorescent proteins, exogenous organic dyes, and semiconductor nanocrystals, among others. A number of versatile fluorescence-based techniques, such as laser scanning confocal, two-photon excited fluorescence, single-molecule microscopy, and super-resolution imaging, have flourished and transformed the way modern life sciences are conducted [2–7].

However, fluorescence microscopy faces two fundamental limitations. First, many chromophores such as hemoglobin and cytochromes absorb light but do not fluoresce efficiently because of their fast nonradiative decay rates from excited states. Second, many intracellular small molecules such as metabolites, signaling peptides, neurotransmitters, and drugs are intrinsically nonfluorescent. It is no longer feasible to label these small molecules with fluorescent labels, because the



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exogenous labels are larger than the sizes of the small molecules of interest. How to probe these nonfluorescent small molecules and chromophores inside live cells and organisms with high detection sensitivity and chemical specificity represents a grand challenge for microscopists. To this end, optical imaging methods other than fluorescence would undoubtedly open up new avenues that will be highly desirable in bioanalytical sciences and applications.

Various forms of pump-probe optical microscopy have been recently developed to visualize the distribution of nonfluorescent but highly absorbing chromophores in biological samples. These techniques are based on fluorescence-free contrast mechanisms, including excited state absorption, stimulated emission, ground state depletion, and the photothermal effect, generated from pump-probe spectroscopy which has been widely employed to study the time-dependent ultrafast phenomena in chemical physics [8, 9]. By using the pump pulse to excite the molecule of interest and the subsequent probe pulse to interrogate the created transient states on a laser scanning microscope, this family of pump-probe microscopy allows imaging of nonfluorescent chromophores with high sensitivity and specificity.

Apparatus

A generic excitation and detection scheme of pump-probe microscopy is depicted in Fig. 1. First, pump and probe beams

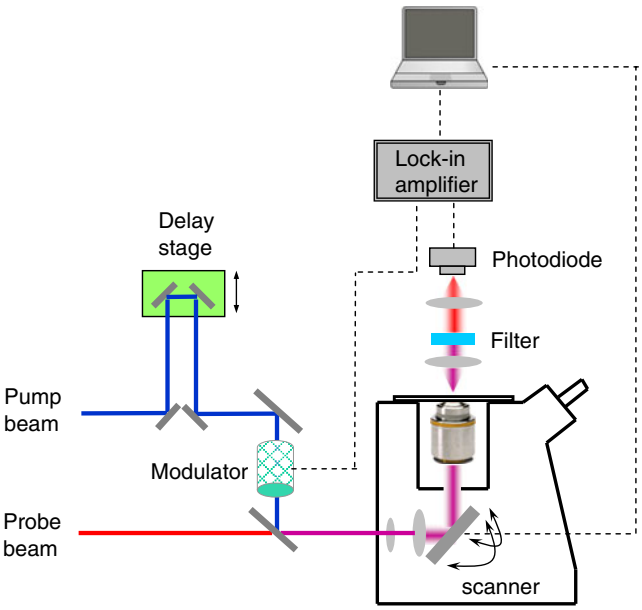

Fig. 1 Apparatus of pump-probe microscopy. After being combined spatially in a collinear manner, both pump and probe beams are focused onto a common focal spot with a microscope objective. An optical delay stage is used to control the relative timing between the pump pulse and the probe pulse. The intensity of the pump beam is modulated at a high frequency (>1 MHz), and probe beam after interacting with the sample is collected and detected by a photodiode and then demodulated by a lock-in amplifier

are normally in the form of ultrafast (femtosecond or picosecond) pulsed lasers in order to exhibit a high time resolution and to be able to capture the transient intermediates for nonfluorescent chromophores, although continuous-wave (CW) lasers are sometimes used in special cases (which will be discussed later in this review). Second, the pump and probe pulse trains are temporally synchronized (with an adjustable time delay) and spatially combined before being focused collinearly onto a common focal spot in the sample. A certain time delay between the pump pulse and probe pulse is often necessary to allow for dynamic evolution of the transient molecular states. Third, the intensity of the pump beam is modulated at a high frequency f (>1 MHz) by an acoustic or electric optical modulator while the probe beam is unmodulated before entering the objective. After interacting with the sample at the common focal volume, only the intensity of the probe beam is collected and detected by a photodiode. The readout of the photodiode is then demodulated by a lock-in amplifier to extract the modulation depth at the frequency f. Fourth, with the amount of the modulation of the probe beam being registered for each pixel, a 3D image is then constructed by scanning the combined pump/probe laser beams across the sample point-by-point with a laser scanning microscope. Although there is no commercial pump-probe microscope system as for now, the individual components (including the lasers, electronics, and detectors) are available off-the-shelf, and assembling them together only requires moderate expertise in optics.

The high-frequency modulation scheme is a crucial technical element for achieving the desirable detection sensitivity of pump-probe microscopy. Laser intensity noise occurs primarily at low frequencies (from kilohertz to DC) in the form of the so-called $1/f$ noise, as shown in Fig. 2a. As f increases above the megahertz range, the laser intensity noise gradually approaches the quantum shot noise floor which is always present due to the stochastic arrival of photons at the photodetector. Thus, the narrowband modulation and subsequent demodulation at f circumvents the low-frequency $1/f$ laser intensity noise and allows for shot-noise-limited detection sensitivity. In addition, as the focused laser beam is scanning across the sample, the intensity variation due to sample scattering will be filtered out by the lock-in amplifier because those variations occur at relatively slow scanning frequencies.

Temporal modulation behaviors of the input and output pump and probe pulse trains before and after interacting with the samples are illustrated in Fig. 2b. Depending on the nature of the underlying light–molecule interactions, the probe beam could undergo either a relative gain or a loss in its intensity. When compared to the originally modulated pump beam at the lock-in amplifier, the probe intensity gain and loss will correspond to an in-phase and anti-phase signal, respectively. Such phase information can be recorded

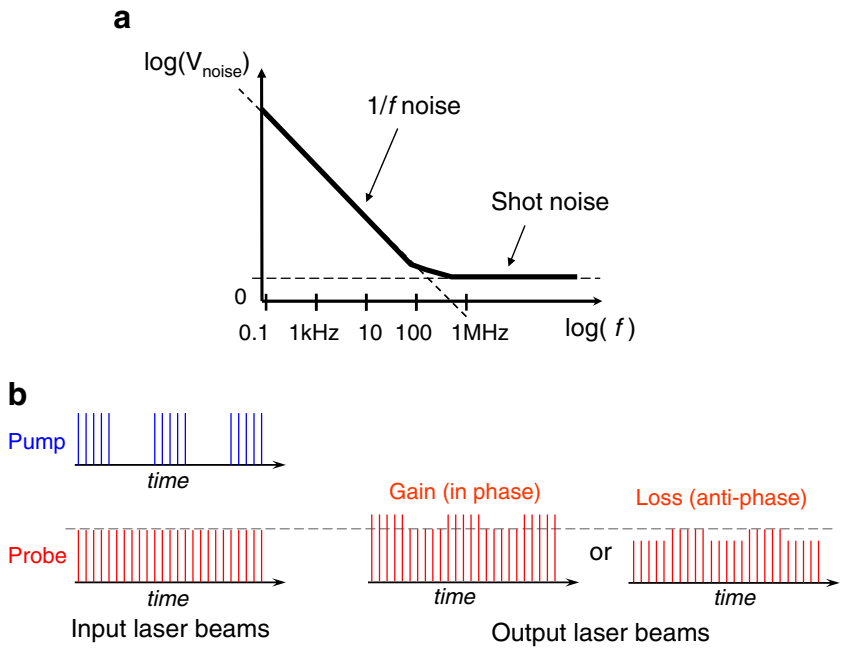

Fig. 2 Principle of high-frequency modulation scheme. **a** Noise spectrum (log–log plot) of a typical laser source as a function of frequency *f*. In the low-frequency range (from DC to kilohertz), the noise follows the so-called 1/*f* noise. In the higher-frequency range, the noise approaches the flat floor of shot noise. The high-frequency modulation scheme creates and subsequently detects the signal at a high frequency *f* (>1 MHz), circumventing the low-frequency laser noise. **b** Temporal modulation behavior of the input and output pump and probe pulse trains before and after interacting with the samples. Depending on the underlying light–molecule interaction, the probe beam could undergo either a relative gain or a loss in its intensity, exhibiting in-phase and anti-phase modulation, respectively

experimentally to allow valuable mechanistic understanding of the probed interaction.

Excited state absorption microscopy

Pump-probe microscopy implemented with the modern high-frequency modulation scheme based on excited state absorption was first reported by Warren's group [10–12]. The pump pulse excites the chromophore to the electronic excited state which can then interact with the probe pulse through further absorption, as shown in Fig. 3a. Without the presence of the pump beam, the probe beam alone does not interact with the chromophore in its ground state. Hence, an intensity modulation of the pump beam at frequency *f* would induce a modulation of the transmitted probe beam at the same frequency *f*. The resulting signal is of lower intensity than the probe beam and is 180° out of phase (i.e., anti-phase) with respect to the original pump beam modulation.

The pump and probe pulses are a few hundred femtoseconds to interrogate effectively the transient excited states, as the excited lifetimes of those nonfluorescent chromophores

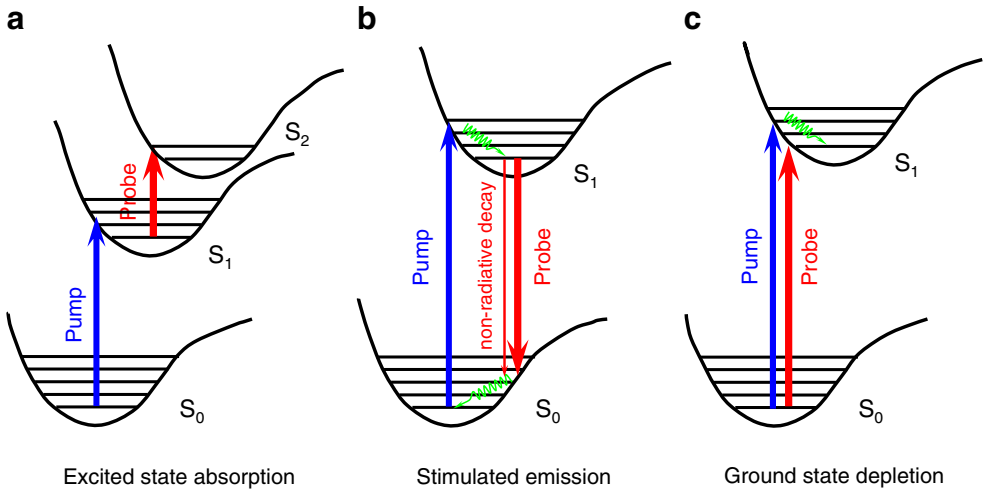

Fig. 3 Energy level diagrams of pump-probe microscopy based on contrast mechanisms of **a** excited state absorption, **b** stimulated emission, and **c** ground state depletion. In all cases, the pump pulse excites the molecule of interest and the subsequent probe pulse interrogates the created transient state(s)

are extremely brief (less than 1 ps). Besides, the pump and probe pulse trains do not need to overlap in time. In fact, the probe pulse train is delayed by a few hundred femtoseconds with respect to the pump pulse train to permit the molecule enough time to relax vibrationally in the electronic excited state. This delay is also useful to separate the excited state absorption signal from other instantaneous nonlinear optical processes such as stimulated Raman scattering [13].

Excited state absorption significantly enhances the detection sensitivity compared to instantaneous two-photon absorption by bringing a resonance with a real intermediate electronic state. For example, ex vivo and in vivo imaging of blood vessels in mouse ears have been demonstrated with 775-nm and 650-nm excitation light by using the charge transfer absorption band of oxyhemoglobin and deoxyhemoglobin in the near infrared. Notably, such an excited state absorption imaging modality presents possibilities for oxygenation imaging based on differences in excited state dynamics between oxyhemoglobin and deoxyhemoglobin [12].

The signal strength, S, defined as the amount of intensity modulation generated to the originally unmodulated probe beam at the frequency f, under the unsaturated condition, is proportional to the product of the pump beam intensity, I_{pump}, the probe beam intensity, I_{probe}, and the analyte concentration, c:

$$S \propto c \times I_{\text{pump}} \times I_{\text{probe}} \quad (1)$$

Because of the overall quadratic intensity dependence, the signal is only generated at the laser focus where the optical intensity is the highest, similar to two-photon excited fluorescence microscopy [3]. Such nonlinearity allows for automatic 3D optical sectioning along the z-axis without the use of a confocal pinhole. This is also the reason why pump-probe techniques are considered as nonlinear optical microscopy [14]. In addition, the linear concentration dependence of the analyte permits straightforward quantification, as opposed to the parametric generation microscopy (such as second harmonic generation, third harmonic generation, and coherent anti-Stokes Raman scattering) which often exhibits quadratic concentration dependence.

Stimulated emission microscopy

Stimulated emission was first theoretically described by Einstein in 1917. An atom or molecule in its excited electronic state can be stimulated down to the ground state by an incident photon with the appropriate frequency, resulting in the creation of a new coherent photon identical to the original incident one in all physical aspects. From the perspective of the molecules, excited state population de-excitation occurs simultaneously with the stimulated emission process. Such a population dumping aspect has been utilized in microscopy, most notably in super-resolution fluorescence imaging by stimulated emission depletion (STED) [6, 15]. From the perspective of radiation fields, stimulated emission represents a process of intensity gaining of the incident field. This is precisely the working principle for light amplification in the laser.

The light-amplification aspect of stimulated emission has recently been demonstrated as a contrast mechanism of pump-probe microscopy for highly sensitive imaging of nonfluorescent chromophores [16]. For those chromophores that absorb light intensely but have undetectable fluorescence, their spontaneous emission is dominated by their fast nonradiative decay (which can be four orders of magnitude faster than the rate of spontaneous emission) from the excited state [17]. As shown in Fig. 3b, with the introduction of a stimulated emission probe pulse with appropriate time delay and matching wavelength, the chromophore, after being photo-excited to the excited state by a pump pulse, is much more likely to be brought down to the ground state through the radiative decay (which consists of spontaneous emission and stimulated emission) channel compared to through the nonradiative decay. As a result of the new photons radiated by the molecule, the intensity of the stimulation probe beam is concurrently increased in-phase, which can be extracted by high-frequency demodulation. A number of applications of stimulated emission microscopy have been demonstrated, such as visualizing chromoproteins, nonfluorescent variants of the green fluorescent protein, monitoring *lacZ* gene expression with a chromogenic reporter, and mapping transdermal drug distributions without histological sectioning [16].

Both the excited state absorption and stimulated emission microscopy exhibit a few nontrivial advantages over direct one-beam absorption microscopy for bioimaging, although they all probe the electronic spectrum of the chromophore. First, the pump-probe signal is only generated at the laser spot, offering automatic 3D optical sectioning. Second, the high-frequency modulation transfer scheme provides shot-noise-limited detection sensitivity, whereas one-beam absorption suffers from laser intensity noise at low frequencies. Third, the one-beam absorption approach cannot distinguish true optical absorption from light scattering from heterogeneous biological samples, as both effects are manifested as light extinction at the detector. In contrast, pump-probe microscopy measures the response of the probe beam intensity only at the pump beam modulation frequency, filtering out the probe beam intensity variations due to sample scattering at low frequencies.

The probe beam modulation in excited state absorption and stimulated emission will exhibit an opposite phase relation with respect to the original pump beam modulation. This phase effect has recently been elegantly utilized as a contrast to discriminate metallic and semiconducting single-

walled carbon nanotubes (SWNTs) [18]. The pump pulse perturbs the electronic state of the SWNT and the probe pulse senses the changed electronic state in the form of stimulated emission or excited state absorption, which gives in-phase and anti-phase signals, respectively. This method holds the potential of serving as a high-speed metallicity-mapping tool to assist the development of SWNT-based nanoelectronics.

Ground state depletion microscopy

Ground state depletion can also be applied to pump-probe microscopy, as shown in Fig. 3c. Unlike excited state absorption or stimulated emission discussed above, ground state depletion employs pump and probe pulses that are both resonant with the absorption band of the chromophore of the ground state. Without the pump pulse, the probe pulse is getting absorbed and attenuated by the chromophores. But after being excited to the higher electronic state by the pump pulse and before ground state recovery, the chromophore would then absorb the subsequent probe pulse to a lesser extent, because of the transient depletion of the ground state population. Hence, the presence of the pump beam will result in a relative in-phase gain of the probe beam intensity compared to absence of the pump beam.

In the case where the excited state is reasonably long-lived, the ground state depletion effect can be readily created by using CW laser beams in a steady state condition. Most recently, this has been employed to detect an absorption signal from single gold nanoparticles and single organic dye molecules in condensed phase at room temperature [19]. The peak amplitude of ground state depletion signal from a single Atto647N dye molecule embedded in poly (methyl methacrylate) film is $\delta\delta P/P \sim 13.5 \times 10^{-8}$, which agrees well with theoretical estimates. Thus, pump-probe microscopy allows the ultimate sensitivity of nonlinear optical microscopy based on saturation spectroscopy: the detection of a single-molecule absorption signal at room temperature.

Photothermal microscopy

Photothermal microscopy, which has long been used for imaging absorbing microscopic objects (especially metal nanoparticles), can be regarded as a special version of pump-probe microscopy [20–24]. In its popular implementation, a pump beam (pulsed or CW) is used to excite the molecules and to induce local heating, and the resulting temperature-dependent refractive index gradient is then read out as a transparent phase object by a second probe beam. Various specific microscopy arrangements have been developed such as thermal lens detection and heterodyne interference [20–24].

Similar to excited state absorption, stimulated emission, and ground state depletion, a high-frequency modulation scheme is often implemented in photothermal microscopy to remove the low-frequency laser intensity noise associated with the probe beam. Most recently, a single-molecule imaging has been demonstrated with impressive signal-to-noise ratio on organic quenchers immersed within glycerol [25].

However, we tend to treat photothermal microscopy differently from the above three pump-probe microscopy techniques. This is so because the probe beam employed in photothermal microscopy does not need to exhibit any direct spectral interaction with any states (ground state or excited state) of the target molecule: its only role is to read out the local refractive index gradient. Thus, a high-power red or near-infrared laser beam, which is off resonant with most optical transitions, is normally used in photothermal microscopy. For example, more than 70 mW of probe beam power was focused into a diffraction-limited focal spot in the recent single-molecule photothermal imaging [25].

Conclusion

The detection sensitivity of excited state absorption microscopy, stimulated emission microscopy, ground state depletion microscopy, and photothermal microscopy has approached the shot-noise limit of the incident laser beam, thanks to the successful implementation of a high-frequency modulation scheme on a laser scanning microscope (Fig. 2). While this is a desirable technical achievement from the point of view of physical measurement, this poses a fundamental challenge as to how to further improve the sensitivity to study even more dilute molecular species or to accelerate the imaging speed. We envision that the principles and techniques from quantum optics, such as entangled photons and squeezed light, might be borrowed to create a "quantum leap" for the detection sensitivity beyond the shot-noise limit.

While the chemical biology strategies of fluorophore labeling have become increasingly sophisticated [26, 27], many molecular species cannot or should not be labeled in biomedical applications. To this end, pump-probe microscopy represents an emerging direction for nonfluorescent optical imaging. Exciting applications in various areas are expected for many years to come.

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