Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Blavatnik Awards for Young Scientists*

What can stimulated emission do for bioimaging?

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Advances in bioimaging have revolutionized our ability to study life phenomena at a microscopic scale. In particular, the stimulated emission process, a universal mechanism that competes with spontaneous emission, has emerged as a powerful driving force for advancing light microscopy. The present review summarizes and compares three related techniques that each measure a different physical quantity involved in the stimulated emission process in order to tackle various challenges in light microscopy. Stimulated emission depletion microscopy, which detects the residual fluorescence after quenching, can break the diffraction-limited resolution barrier in fluorescence microscopy. Stimulated emission microscopy is capable of imaging nonfluorescent but absorbing chromophores by detecting the intensity gain of the stimulated emission beam. Very recently, stimulated emission reduced fluorescence microscopy has been proposed, in which the reduced fluorescence due to focal stimulation is measured to extend the fundamental imaging-depth limit of two-photon microscopy. Thus, through ingenious spectroscopy design in distinct microscopy contexts, stimulated emission has opened up several new territories for bioimaging, allowing examination of biological structures that are ever smaller, darker, and deeper.

Keywords: stimulated emission; superresolution; nonfluorescent chromophore; pump-probe microscopy; deep tissue imaging; imaging-depth limit

Introduction

Light microscopy, since its invention several centuries ago, has played an indispensable role in the life sciences to unveil valuable spatial and temporal information in the study of cells, tissues, and organisms.[1–3] Advances in light microscopy make visualization of live cell composition, dynamics, and physiology possible at a microscopic scale. Among the conceptual and technical factors that have propelled the development of modern light microscopy, stimulated emission is one of the current frontiers. The existence of the stimulated emission process was first theoretically postulated by Einstein back in 1917. It was later confirmed experimentally and now is understood as a universal optical process in which a molecule at its excited state can be stimulated down to its ground state by an incident photon with proper frequency, simultaneously creating a new coherent photon with the same phase, frequency, polarization, and direction as the incident one. Figure 1 illustrates the competition between stimulated emission and spontaneous emission (i.e., fluorescence) processes.

The first and arguably the most notable application of stimulated emission in bioimaging is stimulated emission depletion (STED) microscopy for breaking the diffraction-limited spatial resolution of lens-based far-field fluorescence microscopy. Since its original proposal in 1994, STED has extended fluorescence microscopy to nanoscopy.[4,5] Going beyond the popular fluorescence contrast, stimulated emission was applied in 2009 to the detection and imaging of absorbing chromophores with nondetectable fluorescence using pump-probe optical techniques.[6] Most recently, inspired by STED microscopy and stimulated emission microscopy, stimulated emission reduced fluorescence (SERF) microscopy was proposed to extend the fundamental imaging-depth limit of two-photon fluorescence microscopy inside highly scattering samples.[7]

doi: 10.1111/nyas.12079

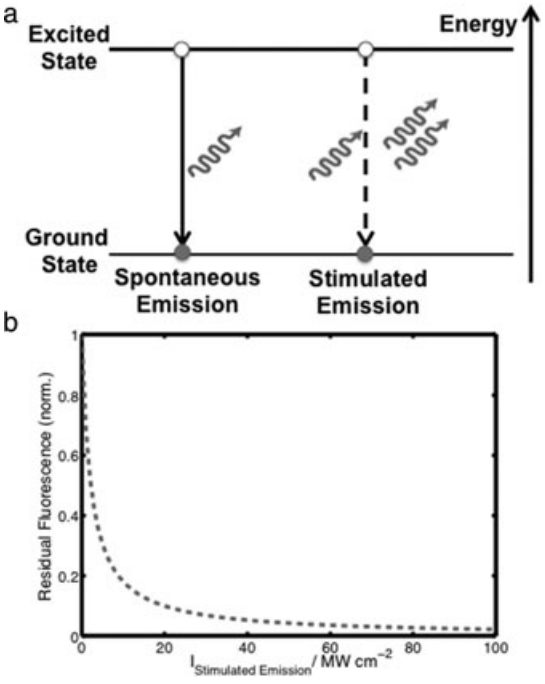

Figure 1. Comparison between the spontaneous emission and the stimulated emission. (A) For spontaneous emission, upon excitation, the molecule will relax from its excited state back to its ground state and concurrently emit a fluorescence photon at a certain frequency with a random phase and direction. In contrast, in stimulated emission, the molecule, after being excited to its excited state, also experiences the incoming photon(s) whose energy matches the energy gap between its excited and ground state. This molecule is then brought back to the ground and emits a photon exhibiting the identical physical properties as the incident stimulated emission photons. (B) Depletion of the fluorescence from a typical fluorophore as a function of the laser intensity I of a continuous-wave stimulated emission beam, illustrating the competition between the spontaneous emission and the stimulated emission.

Thus, when applied in different microscopy contexts, stimulated emission has opened up several frontiers of bioimaging, allowing one to look at target structures that are much smaller, darker, and deeper than previously possible. We will review these three techniques individually before summarizing their underlying interconnections.

Breaking the diffraction-limited spatial resolution

In lens-based far-field fluorescence microscopy, diffraction-limited spatial resolution (∼200 nm) has been a serious issue for over a century.[8] Because light cannot be focused tighter than its diffraction limit, the image of an object that is smaller than the diffraction limit inevitably becomes blurry. As a result, the ability to clearly resolve fine biological structures smaller than 200 nm was exclusive to electron microscopy, which is, however, not compatible with live imaging. Since its original theoretical description in 1994[4] and first experimental demonstration in 1999,[9] STED microscopy has achieved lateral resolution of about 15–20 nm in biological samples, which is a 10- to 12-fold increase over diffraction-limited resolution,[10] opening up the entire super-resolution field for resolving structures that are too blurry for conventional fluorescence microscopy.

STED microscopy typically adopts a confocal scheme, using a tightly focused excitation laser spot, collinearly combined with a doughnut-shaped and red-shifted STED laser beam (Fig. 2A) to scan across the sample plane. The rationale behind STED is the selective deactivation of fluorophores at the edge of a laser spot via stimulated emission depletion, thereby allowing only the fluorophores at the very center to fluoresce[11] (Fig. 2B). By elevating the intensity of the doughnut-shaped STED beam to saturate the stimulated emission process, resolution can be continuously reduced into a progressively fine scale, extending the classic Abbe's diffraction limit of $d = \lambda/(2NA)$ to a new diffraction-unlimited regime of $d \approx \lambda/(2NA\sqrt{1 + I/I_s})$, where d is the full-width-at-half-maximum (FWHM) of the focal spot at the focal plane, λ is the excitation wavelength, NA is the numerical aperture, I_s is the intensity at which half of the fluorophores are quenched (i.e., loss of fluorescence due to stimulated emission), and I is the applied intensity of the doughnut laser beam.[12]

In recent years, STED has matured into a popular and widely used superresolution technique, especially with the advent of continuous wave (CW) STED.[13] Structural analysis of the structures and distributions of proteins such as tubulin and other cytoskeletal filaments on suborganelle levels has become standard using STED microscopy.[14] Multicolor STED has also been made possible, from its first demonstration on colocalization imaging of synaptic and mitochondrial protein clusters with 5 nm precision[15] to more complicated analyses, such as the analysis of protein–protein interaction in parallel channels.[16,17] Moreover, with the improvement of genetically encoded fluorescent

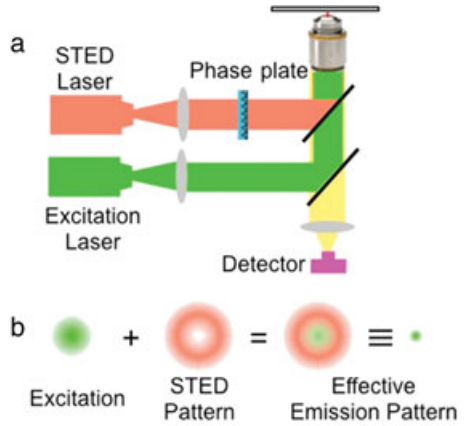

Figure 2. Setup and principal illustration of stimulated emission depletion (STED) microscopy. (A) Basic setup of STED microscopy. A spatially shaped doughnut STED beam created by using a phase plate is collinearly combined with an excitation beam. Both beams are tightly focused onto the sample. A detector detects the residual fluorescence signal in the presence of the STED laser beam. (B) An illustration of the pattern of each focused laser beam onto sample as well as the final effective excitation profile achieved by the STED design. The diffraction-limited excitation beam (green) is overlapped with the doughnut-shaped STED beam (red) that quenches the fluorescence at the edge of the excitaton beam. The remaining fluorescence is only generated at the center of the excitation beam, which effectively results in an overall narrower emission pattern.

proteins,[18,19] live-cell STED microscopy has offered rich and valuable information. For example, the time-lapse STED imaging of both dendritic spine cells[20] and living mouse brains[21] exhibit important structural details. Additionally, STED imaging of cultured hippocampal neurons illustrates that endosomal sorting of synaptic vesicles is a rapid pathway.[22] In addition, examination of membrane lipid dynamics at nanoscale reveals that unlike phosphoglycerolipids, sphingolipids and glycosylphosphatidylinositol-anchored proteins are transiently (∼10–20 ms) trapped in cholesterol-mediated molecular complexes dwelling within areas of less than 20 nm diameter.[23] Efforts have also been devoted to enable video-rate STED (∼28 frames/s),[24] which is important for imaging dynamic processes such as synaptic plasticity. Furthermore, in order for deep tissue superresolution imaging, aberration-reducing optics have been utilized to demonstrate a resolution of 60–80 nm in living organotypic brain slices at depths of up to 120 μm.[25] Meanwhile, two-photon excitation in combination with CW STED beam has also been demonstrated to be feasible[26] and has been applied to image brains slices with a threefold resolution increase at below 100 μm depth.[27]

STED microscopy is undoubtedly a milestone in the development of advanced fluorescence microscopy. Its robust and general spectroscopic mechanism and intrinsic compatibility with scanning confocal and multiphoton microscopy make it widely useful in various fields of biomedical sciences. However, because of the high stimulated emission laser intensity, a certain degree of photodamage on the samples is inevitable. In addition, one certainly has to acquire some optics expertise before building a complex STED microscope, but once built, it can be used as easily as a confocal microscope.[11]

Imaging nonfluorescent but absorbing chromophores

In addition to its ability to increase spatial resolution of fluorescence microscopy for a sharper visualization of what is being seen, stimulated emission has also been applied to detection and imaging of nonfluorescent but absorbing chromophores to explore what appears to be invisible.[6] There exist many chromophores in life systems that absorb heavily but have undetectable fluorescence, such as hemoglobin and cytochromes, because of their short excited state lifetimes (<1 ps) due to their rapid nonradiative decay processes over spontaneous emission.[28] Unfortunately, direct one-laser absorption microscopy has low sensitivity. Hence, imaging these nonfluorescent but absorbing molecules with sufficient sensitivity has been rather challenging, especially in complex biological samples.

Stimulated emission microscopy adopting a high-frequency modulation transfer scheme provides a suitable solution for the above challenge. Figure 3 shows a cartoon of the stimulated emission microscopy setup and its signal generation process. A pulsed (pulse width ∼200 fs) excitation laser beam is spatially overlapped and temporally synchronized with another pulsed (∼200 fs) stimulated emission beam whose wavelength is properly red shifted. A few hundred femtoseconds of time delay between these two pulse trains is chosen to prevent the occurrence of instantaneous optical processes, such as, for example, stimulated Raman scattering[29,30] and two-photon absorption.[31]

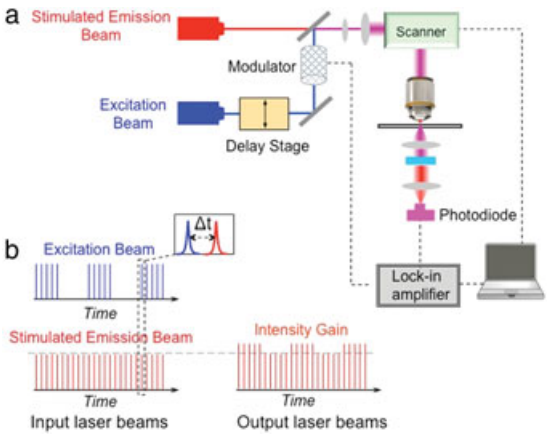

Figure 3. Cartoon demonstration of a stimulated emission microscopy setup for imaging nonfluorescent chromophores. (A) A modulated excitation laser beam is collinearly aligned with a stimulated emission beam before being sent into the sample. The intensity gain of the transmitted stimulated emission beam is then demodulated by a lock-in amplifier at the modulation frequency to ensure shot-noise limited detection sensitivity. (B) Temporal characterization of the input and output beams. The modulated excitation pulse train (blue) is synchroized with the stimulated emission pulse train (red) with a temporal time delay (∼300 fs) between these two pulse trains. With the excitation beam on, the stimulated emission pulse train experiences an intensity gain; with the excitation beam off, the stimulated emission pulse train intensity remains unchanged after interacting with the sample.

In the common focal volume, after being excited by the excitation pulses, the chromophores are subsequently interrogated by the stimulated emission pulses, which bring them down to the ground state faster than the nonradiative process. As a result, after passing through samples, the intensity of the stimulated emission beam will be increased due to photons newly created by the chromophores. However, the relative intensity gain is normally small ($<10^{-3}$) that can easily be buried by the low-frequency laser noise. To achieve the necessary detection sensitivity, a high-frequency modulation transfer scheme is employed, where the excitation beam intensity is modulated by an acousto- or electro-optic modulator at high frequency (>1 MHz) and the transmitted stimulated emission beam is demodulated by a lock-in amplifier at the same modulation frequency to extract the intensity gain and to reject noises at other frequencies.

Stimulated emission microscopy has been successfully applied to visualizing chromoproteins (the nonfluorescent variants of the green fluorescent protein; GFP) in live *Escherichia coli* cells, monitoring lacZ gene expression with a chromogenic reporter, and mapping transdermal drug distributions without histological sectioning.[6] The detection limit for stimulated emission microscopy is 60 nM for crystal violet with one-second integration time. This sensitivity effectively corresponds to a few (∼5) molecules in focus.[6] The advantages of stimulated emission microscopy include: (1) because of its signal dependence on both excitation and stimulated emission laser intensities, its nonlinear nature offers intrinsic 3D sectioning ability; (2) the high-frequency modulation scheme ensures shot-noise limited detection sensitivity by getting rid of the lower frequency laser noise; and (3) the modulation transfer between two laser beams (in comparison with traditional one-beam absorption microscopy) avoids undesired signal artifacts from heterogeneous sample scattering.[32] These features make stimulated emission microscopy a desirable technique for imaging chromophores with high sensitivity and specificity in complex biological environments. The major complication for stimulated emission microscopy is the difficulty of synchronizing the two-femtosecond laser pulse trains.

Extending the fundamental imaging-depth limit

It is generally believed that the spatial resolution and the penetration depth of a given imaging modality are inversely correlated. For example, MRI has a poor spatial resolution but superb penetration depth. In the domain of light microscopy, while the diffraction-limited spatial resolution barrier has been broken by STED, photoactivated localization microscopy (PALM), and stochastic optical reconstruction microscopy (STORM),[4,5,33,34] the deepest penetration into scattering samples with subcellular resolution is achieved currently by two-photon fluorescence microscopy.[35] By employing a nonlinear optical excitation, two-photon fluorescence is primarily generated at the focal volume. Such a spatially confined excitation scheme thus permits the capture of fluorescence photons emitted and then scattered from the focus by a nondescanned detector, dramatically increasing the detection sensitivity. This profound advantage of two-photon imaging leads to an imaging depth that is more than three times deeper than what can be achieved with confocal microscopy.

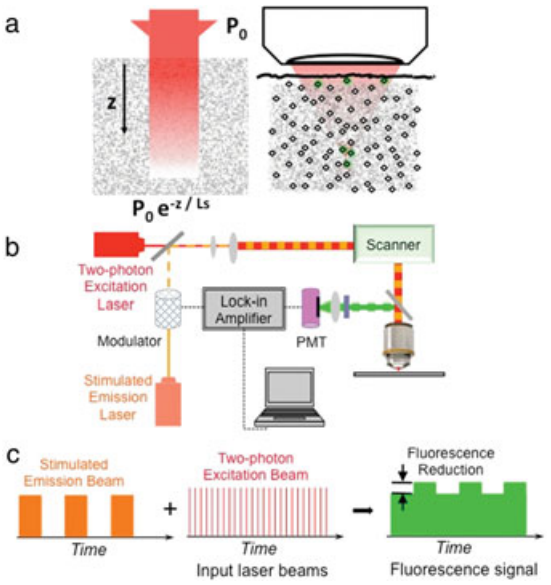

Figure 4. Extending the fundamental imaging-depth limit of two-photon microscopy by stimulated emission reduced fluorescence (SERF) microscopy. (A) Illustration of the fundamental imaging depth limit of two-photon microscopy in the scattering tissue sample. Incident light power decreases exponentially into the scattering sample. Thus, the fundamental imaging depth limit of two-photon microscopy is defined as the depth at which the in-focus signal and the out-of-focus background signal are equal. (B) The proposed SERF microscope setup. A modulated stimulated emission beam is combined collinearly with a two-photon excitation beam. Then, the reduced fluorescence due to quenching is measured by a lock-in amplifier and used to form SERF images. (C) The modulation transfer scheme of SERF. The CW stimulated emission beam is modulated at a high frequency (> MHz). When combined with the two-photon excitation pulse train, this modulated stimulated emission beam leads to the resulting two-photon fluorescence signal modulated at the same frequency.

However, a fundamental imaging-depth limit still exists for two-photon microscopy. For example, for mouse brain tissues labeled with GFP, the corresponding depth limit is about 1 mm.[36] Such a depth limit is not constrained by the available laser power, but instead by the achievable image contrast.[36–38] When imaging highly scattering samples, with the increase of focusing depth, the laser power has to be elevated accordingly in order to compensate for scattering loss. At some point (Fig. 4A), the laser power deposited at sample surfaces becomes so strong that it generates comparable or even stronger fluorescence than that from the focal volume, which thus deteriorates the achievable image contrast. Formally, the depth where the in-focus signal and the out-of-focus background are equal to each other is defined as the fundamental imaging-depth limit.[36,38] Thus, the conventional optical sectioning picture that two-photon fluorescence is generating only within the focal volume breaks down here. Obviously, further increasing laser power cannot extend this imaging-depth limit.

One plausible strategy to extend the fundamental imaging-depth limit is to suppress the out-of-focus background as recently demonstrated by focal switching of optical highlighter fluorescent probes.[39–41] This strategy requires the use of special fluorescent probes, which can sometimes be inconvenient. To be generally applicable to all fluorophores, stimulated emission reduced fluorescence (SERF) microscopy is proposed to introduce an additional stimulated emission process to distinguish the desired focal signal from the out-of-focus background:[7] when the stimulated emission laser is focused collinearly with a two-photon laser, the stimulated emission process will preferably occur at the focus because the intensity of the stimulated emission beam at the focus is much higher than its out-of-focus counterpart. Figure 4B depicts the proposed SERF microscope setup. The technical aspect of SERF is analogous to optical lock-in detection (OLID)[42] and synchronously amplified fluorescence image recovery (SAFIRe)[43] techniques; however, OLID and SAFIRe tackle problems related to autofluorescence background.

By spatially overlapping the two-photon excitation laser (pulse train) with an intensity-modulated and red-shifted CW-stimulated emission laser (pulsed laser works equally well, but is technically more demanding), the two-photon excited fluorescence is collected and then demodulated with a lock-in amplifier at the modulation frequency. Instead of detecting the residual fluorescence, as in STED, or the transmitted stimulated emission beam, as in stimulated emission microscopy, SERF effectively detects the fluorescence signal reduction (Fig. 4C) due to stimulated emission–based quenching. Thus, SERF combines the fluorescence quenching mechanism, as in STED, and the high-frequency modulation transfer scheme, as in stimulated emission microscopy. It can be quantitatively demonstrated that, at the weak fluorescence quenching region (i.e., where the fluorescence signal reduction has a linear relationship to the applied stimulated emission laser intensity), the final SERF

signal exhibits an overall three-photon dependence,[7] including the original two-photon excitation and the new one-photon stimulated emission. As shown by the numerical simulation,[7] such a higher order nonlinearity (compared to the standard second-order effect) offers a much higher signal-to-background ratio and a contrast-limited imaging-depth limit that is effectively 1.8 times deeper. The disadvantage of SERF lies mainly in its imaging speed: because of the relatively moderate signal size deep inside scattering samples, a relatively long integration time may be needed for acquiring a decent signal-to-noise ratio.

Conclusion

We now summarize the interconnection of the above three stimulated emission–based bioimaging techniques. There exist three total measurable physical quantities in the stimulated emission process: (1) the residual fluorescence; (2) the reduced fluorescence; and (3) the enhanced stimulated emission beam. As illustrated in Figure 5, each of the techniques discussed above measures one of these three quantities to accomplish the respective goal. To squeeze the effective emission pattern, STED measures the residual fluorescence in the center of the focal spot quenched by the doughnut-shaped stimulated emission depletion beam. To generate an optically detectable signal from nonfluorescent chromophores, stimulated emission microscopy measures the enhanced intensity of the transmitted stimulated emission beam. To create even higher order nonlinearity on top of two-photon excited fluorescence, SERF measures the reduced fluorescence by introducing an additional stimulated emission laser beam.

Since both STED and SERF deal with fluorescent molecules, it is highly constructive to compare the technical aspects of these two. Both techniques harness the fluorescence quenching process of stimulated emission. However, STED aims to break the spatial resolution limit while SERF is designed to extend the penetration depth. It is interesting to see how stimulated emission can contribute to both ends. Third, the stimulated emission beam in STED is spatially shaped, whereas it is being temporally modulated in SERF. Finally, STED works best in the fluorescence depletion region where the stimulated emission intensity is strong, while SERF operates in the nonsaturating region where the stimulated emission intensity is moderate in order to perform deep imaging.

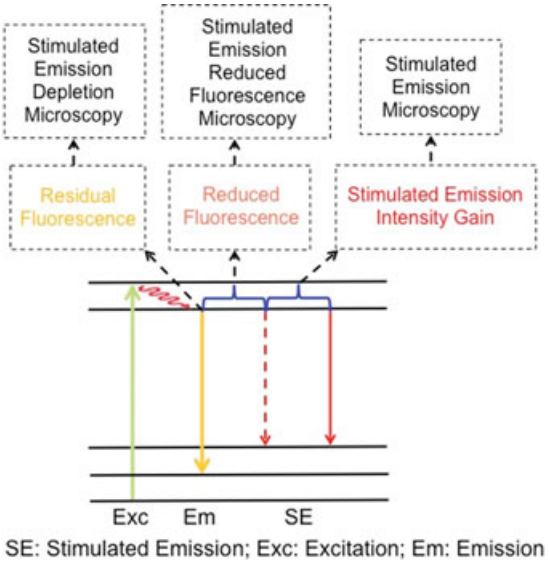

Figure 5. Diagram of physical quantities in the stimulated emission process that are being used in the three imaging techniques reviewed here. In the presence of stimulated emission, the three measurable optical quantities are the residual fluorescence; the reduced fluorescence; and the enhanced stimulated emission beam. Stimulated emission depletion microscopy (STED) makes use of the residual fluorescence to improve the spatial resolution problem; stimulated emission reduced fluorescence microscopy detects the reduced fluorescence to extend the fundamental imaging-depth limit of two-photon microscopy; and stimulated emission microscopy detects the intensity gain of the transmitted stimulated emission laser beam to image nonfluorescent chromophores.

There is no doubt that the universal stimulated emission principle has played a significant role in driving the development of advanced bioimaging techniques, allowing one to look at target structures that are much smaller, darker, and deeper than previously possible. It is rather striking to see how it can be applied in distinct microscopy contexts to bring novel solutions to seemingly unrelated problems. More exciting biomedical applications in various areas are expected for many years to come.

Acknowledgments

The authors thank Zhixing Chen and Rafael Yuste for helpful discussions. W.M. acknowledges the start-up funds from Columbia University, and grant support from the Kavli Institute for Brain Science.

Conflicts of interest

The authors declare no conflicts of interest.

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