

Transient Absorption: A New Modality for Microscopic Imaging of Nanomaterials in Living Cells

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Dedicated to Professor Qing Huang

Transient absorption is a secondary absorption that happens after a material has been excited through primary absorption. Different mechanisms can contribute to transient absorption. This universal photophysical process exists in almost all types of nanomaterials, making it an ideal modality to monitor the location, dynamics, and interactions of nanomaterials in living cells, tissues, or animals. With two beams of lasers and a scanning microscope, transient absorption microscopy is able to acquire high-resolution, 3D images at high speed, without the need for labeling. Through time-delay adjustments of pulse trains, this novel method can also reveal background-free images of specific nanomaterials, even with the interference of high concentrations of fluorophores.

Nanomaterials have long been considered to have profound effects, both good and bad, on biological systems. The major goal to incorporate nanomaterials into biological systems is to implement new functions and the better performance of these materials. Many nanomaterials, especially nanoparticles of different compositions, have been used as molecular probes, labeling reagents, as well as carriers for drug delivery. Some nanoparticles, such as gold nanoparticles and quantum dots, have been widely used in many bioassays for point-of-care tests because of their superior optical properties including distinct colorimetric responses, high photostability, high fluorescence quantum yields, and narrow optical bandwidths.^[1,2]

Most nanomaterials can be easily delivered into living cells. However, the behavior of nanomaterials in living cells is

always more complex than expected. It is critical to unveil the interaction details between nanomaterials and cells, in order to better understand the biological consequences resulting from these artificial perturbations. A few popular cellular uptake processes, including endocytosis, phagocytosis, pinocytosis, and macropinocytosis, have been closely linked to nanomaterial internalization.^[3] Most of these processes happen very quickly in physiological conditions. There are two major 'common practices' to use nanomaterials in cellular biology studies: 1) to implement nanomaterials as specific imaging probes to monitor the bioactivity inside living cells; 2) to use nanomaterials as stimuli to trigger biological responses of living cells, and to observe the cell activity to assess the biocompatibility of nanomaterials. Both applications rely highly on spatial information provided by high-resolution microscopic imaging technologies.

When nanomaterials are used as imaging probes, fluorescence/ luminescence is the major modality for cellular microscopic imaging. For cellular imaging, three dominant types of luminescent nanomaterials are quantum dots, metal nanoparticles, and fluorophore-coated nanoparticles. With special configurations, even the motion and orientation information of nanoscale matter can also be extracted from far-field imaging data.^[4-7] All these particles can be further modified to specifically bind to certain biomarkers or certain subcellular organelles. With proper preparation, nanoparticles can also be implemented to super-resolution imaging, with improved stability and robustness.^[8] However, nonfluorescence optical

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modalities such as Raman scattering,^[9] nonlinear optical harmonic generation,^[10] ground-state depletion,^[11] and excited-state transient absorption^[12] have shown unique advantages over fluorescence in many bioimaging applications. These optical modalities provide intrinsically high spatial resolution, mostly in 3D, that fits well to cell biology studies, as well as the potential medical applications.

Transient absorption is a transition between short-lived electronic states. It can be attributed to various photophysical processes, including two-color, two-photon absorption, stimulated emission, excited-state absorption, and ground-state depletion (**Figure 1**). In this Concept article, we focus more on excited-state absorption. Transient absorption has been recently demonstrated as a suitable modality for imaging many kinds of nanomaterials in living cells and animals. Transient absorption microscopy is becoming a good complement to fluorescence-based imaging modalities.

Transient absorption has been studied for decades, mostly as a spectroscopic tool to investigate the dynamics of certain electronic states, commonly excited states, which only exist for very short period of time – a transient event.^[13,14] Two light beams are usually needed to capture this process (Figure 1). A pump beam is applied to introduce the primary transition between the ground state and the first excited state. In most experiments, this primary transition is a linear optical absorption which can also be well characterized by a normal absorption spectrum. The lifetime of the excited state is in the range from picoseconds to microseconds, depending on the intrinsic nature of the excited state, the transition probability, and the surrounding environment of the fluorophores or particles (e.g., solvation effects). Through this transition, the ground state population will first decrease and then recover through the deactivation of the excited state. The absorption of the material will be different upon this population rearrangement between the electronic states, the absorption of ground state will be reduced while a new absorption of the excited state (secondary absorption) may appear in the spectrum. If no other electronic states are involved, the recovery of the ground state absorption usually will match well with the decay of the excited state absorption.

In contrast to the fact that many materials are not fluorescent, almost all materials can absorb light at a certain wavelength, making absorption-based detection a universal method to generate contrast for bioimaging. However, neither primary absorption nor transient absorption is likely to generate comparable imaging contrast to fluorescence. Hence, for those nanomaterials with strong photoluminescence, confocal microscopy and multiphoton fluorescence microscopy will probably still be the first choices to monitor their distribution inside cells. Nevertheless, fluorescent nano-labels also have their limitations. Many quantum dots, with superior photoluminescence efficiency, can hardly be used in clinical applications due to their toxicity. The excitation and emission wavelengths of many fluorescent nanoparticles also largely overlap with the autofluorescence of cells, leading to the greatly suppressed contrast of images. Relatively short wavelengths of excitation also cause a higher risk of photodamage and phototoxicity.

Recently developed transient absorption microscopy allows us to overcome these limitations with an alternative



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approach. Unfortunately, at this moment, there is not any commercially available transient absorption microscope on the market. All the available systems are modified from maturely designed confocal or multiphoton scanning microscopes.^[15–18] In a typical transient absorption microscope (**Figure 2**), two lasers with different wavelengths, the pump and probe lasers, are introduced into the scanning unit. The two beams are co-linearly aligned, with proper adjustment to make sure the focal points overlap in the space. The lasers can be either pulsed or continuous. The transient absorption signal is the differential absorption, which can be challenging to detect if the difference is not significant compared with the fluctuation of laser intensity. Lock-in amplification,^[15] widely used in spectroscopic studies, is a powerful technology to pick the weak signal from a highly variable background. If the primary absorption is modulated, the secondary absorption will also be modulated at the same frequency. Through modulation, the detection sensitivity, in terms of intensity change in the probe beam, can reach 10^{-7} ,^[9] which is far more than enough for transient absorption measurements. Hence, a moderate laser power can be applied to living cells, which consequently causes less photodamage.

Transient absorption microscopy has been applied to study various types of nanomaterials.^[19] Although, in many cases, the major goal of the study is to investigate the structure and evolution of vibrational or electronic states,^[20–24] this universal applicable method can be seamlessly integrated into biological studies to identify specific nanoscale objects inside the living cells or animals.

Carbon nanomaterials are specifically important and unique for biomedical applications. One of the major

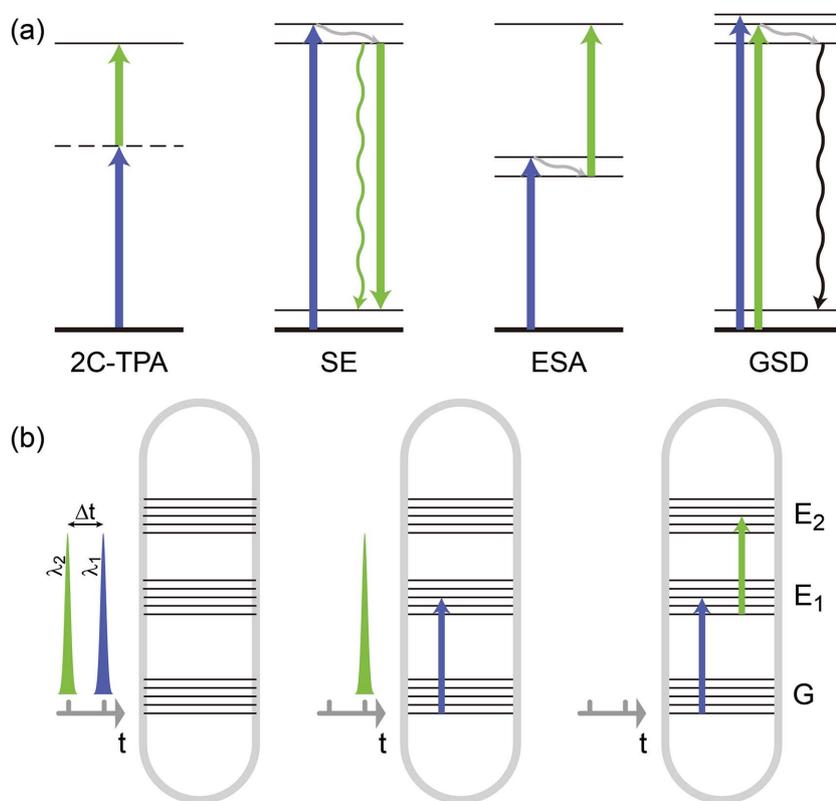


Figure 1. Energy-level diagram of transient absorption processes. a) Possible photophysical contributions to transient absorption. Solid lines represent real energy states, while dashed lines represent virtual states. Thick and thin lines represent vibrational ground and excited states, respectively. Blue and green arrows represent photons with an energy of hc/λ_1 and hc/λ_2 , respectively. Wavy arrows represent spontaneous emission. (2C-TPA: two color two photon absorption; SE: stimulated emission; ESA: excited state absorption; GSD: ground state depletion.) b) ESA process in a nanoparticle. A pump pulse at λ_1 causes the transition between the ground state and first excited state, while a probe pulse at λ_2 , delayed by Δt , leads to further transition to a higher-level excited state. (G: ground state; E_1 : first excited state; E_2 : a higher-level excited state.)

attractions of carbon nanomaterials is that they seem to have relatively better biocompatibility in terms of toxicity and inertness.^[25] Nanotubes and nanodiamonds have been extensively studied to explore their capability as drug carriers and as imaging probes.^[26,27] To visualize these nanoscale objects lacking sufficient luminescence efficiency, various groups (including ours) have employed transient absorption microscopy to image carbon nanotubes^[28] and nanodiamonds^[29] in living cells with an optical 3D-sectioning capability and diffraction-limited resolution.

Carbon nanotubes have long been considered a model system to study the interactions between cells and 1D nanomaterials. To visualize the location of nanotubes, besides the intrinsic photoluminescence,^[30] fluorescent dyes with proper tags have been developed to conjugate to the nanotubes by covalent or noncovalent methods.^[31] In 2010, J.-X. Cheng's group reported a fast and contact-free imaging method to differentiate single-wall metallic carbon nanotubes from semiconducting ones using transient absorption microscopy.^[15] It did not take them too long to apply this new modality to observe the nanotubes in living cells, tissues, and animals (**Figure 3**).^[28] Compared with other optical imaging modalities, transient absorption can effectively separate the background signal from red blood cells, with a unique function to simultaneously

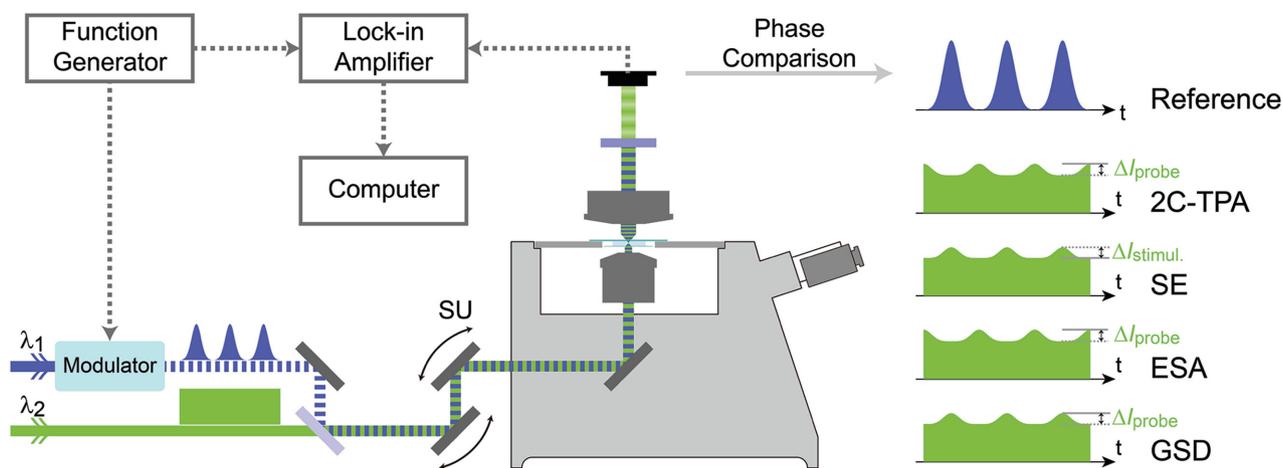


Figure 2. A typical configuration of a transient absorption microscope. Two lasers, pulsed or continuous, with different wavelengths, λ_1 and λ_2 , are spatially overlapped and co-linearly introduced into the microscope. One of the beams, λ_1 , is modulated. In the microscope, a set of galvo scanning mirrors reflects the beams through a region of the sample at the focal plane. Transmitted light is collected and filtered, then projected onto a reversely biased photodiode. With a lock-in amplifier, the change in absorption of λ_2 is detected. A comparison of different phases of possible signals in such a setup is presented. (SU: scanning unit; 2C-TPA: two-color two-photon absorption; SE: stimulated emission; ESA: excited state absorption; GSD: ground state depletion.)

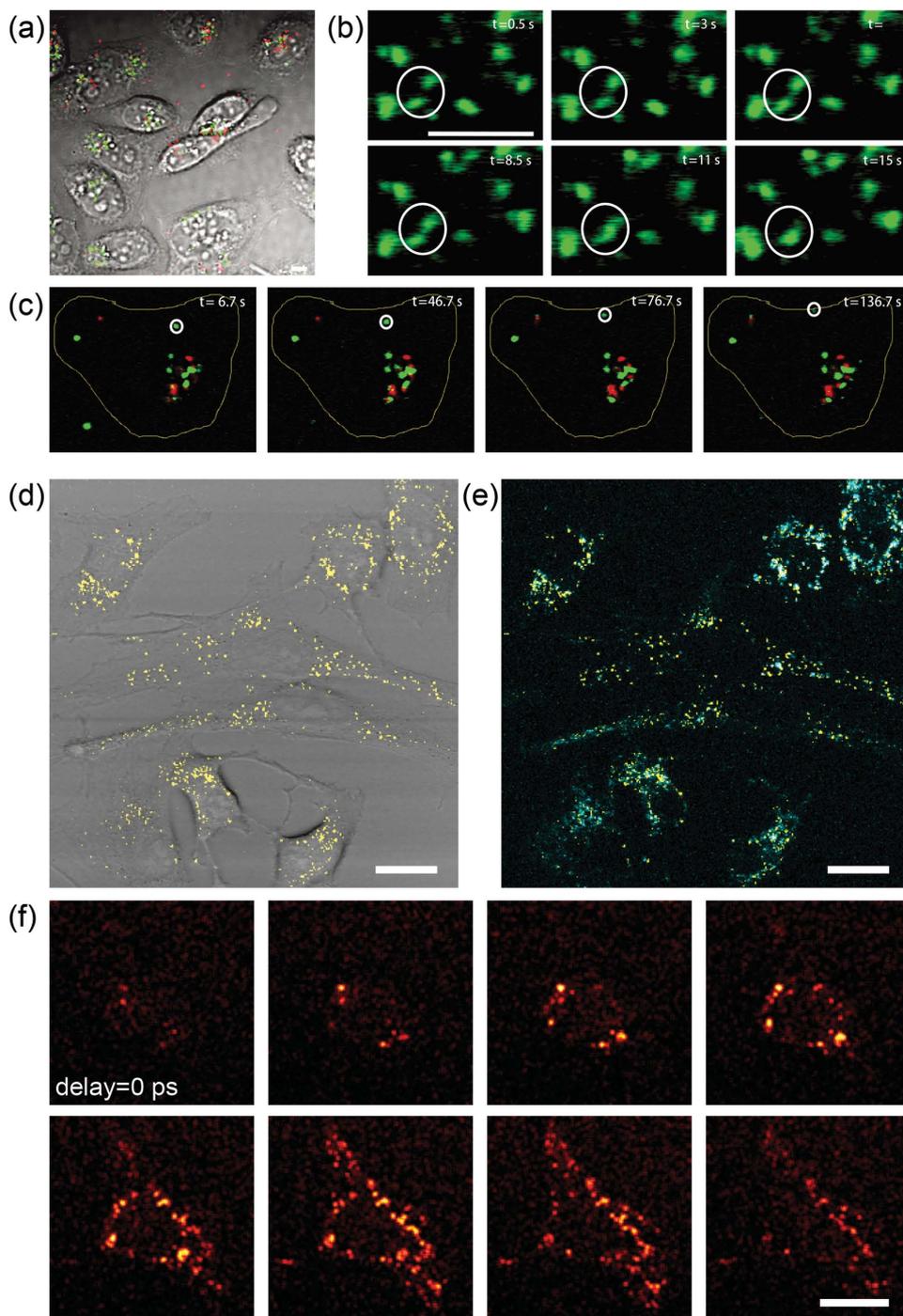


Figure 3. Representative transient absorption microscopic images of single-wall carbon nanotubes (SWNTs) and nanodiamonds (NDs) in living cells. a) An overlay of SWNTs (green and red) and a bright-field image of CHO cells. b) A fusion event of two nanotubes recorded by time-lapse transient absorption imaging. c) Time-lapse images showing the transport of a nanotube to a cell surface. Panels (a–c) reproduced with permission.^[28] Copyright 2012, Macmillan Publishers Ltd. d) An overlay of NDs (yellow) and a bright-field image of Hela cells. e) An overlay of NDs (yellow) and fluorescence-labeled endocytosis vesicles (cyan). f) 3D transient absorption microscopic images taken from eight successive focal planes with 1 μm step. Panels (d–f) adapted with permission.^[29] Copyright 2013, The Royal Society of Chemistry. Scale bars: 5 μm in (a–c), 20 μm in (d–f).

monitor both metallic and semiconducting single-wall carbon nanotubes. It is worth pointing out that the transient absorption signal may coexist with other nonlinear optical signals such as stimulated Raman scattering or ground state depletion (which can also be considered a special case of transient

absorption, since both pump and probe beams detect the same transition). One way to avoid the conflict between these modalities is to carefully choose the proper wavelengths, and the other way to differentiate the modality is to check the phase information of the signal (Figure 2).

Nanodiamonds, a type of inert nanoscale carbon particle with a diamond structure, have shown the least cytotoxicity among carbon nanomaterials. By introducing negatively charged nitrogen vacancy centers into the lattice, nanodiamonds become photoluminescent.^[32] We have shown that those nonfluorescent, raw nanodiamonds can be clearly visualized, in 3D, in living cells, using transient absorption microscopy (Figure 3).^[29] The signal intensity of transient absorption is orders of magnitude higher than for Raman scattering, even over 10-fold higher than stimulated Raman scattering, making it an ideal method to monitor the dynamic behavior of nanodiamonds and their interactions with cells. Single clusters of particles can be monitored when cell uptake happens. The intrinsic 3D sectioning capability of transient absorption microscopy allows us to precisely follow the dynamic process of endocytosis. Other label-free methods will not be able to capture such events due to the long imaging time for each frame. Our TAM obtained a frame rate of 2.45 s per frame at an image size of 320 × 320 pixels. In comparison, for example, it would take more than 20 min for a spontaneous Raman microscope to map a 128 × 128 pixel image. By tuning the two pulse trains to overlap in time, the faint background stimulated Raman scattering can be employed to indicate the contour of the cells. While the pulse trains are slightly tuned off in time, this background vanishes and nanodiamonds distinguish themselves through the dominant transient absorption signal.

Besides carbon nanomaterials, metal nanoparticles such as gold nanorods have also been extensively investigated for their potential biomedical applications. Unlike raw nanodiamonds, most well-engineered gold nanorods with suitable dimensions have great photoluminescence properties.^[33] This plasmonic emission offers great contrast for fluorescence observation. However, the emission spectrum of gold

Table 1. Summary of nanomaterials studied by transient absorption.

Nano-material	TA modality	Ref.
Gold nanoparticles	GSD	[35]
Gold nanorods	ESA	[34]
Nanodiamonds	ESA	[29]
Single-wall carbon nanotubes	SE, Pump-induced spectra broadening	[15,18,28]

nanorods may overlap with cellular autofluorescence or other exogenous fluorophores. We demonstrated that, as expected, gold nanorods have a strong transient absorption signal which decays much slower than that of nanodiamonds (Figure 4).^[34] Using transient absorption as a contrast, we have been able to follow each single nanorod in the cell, and to identify it with a strong fluorescence background produced by two-photon excitation in the imaging conditions. This unique specificity of transient absorption may greatly reduce the engineering efforts that have been invested to avoid spectral conflict.

Although in its infancy, transient absorption microscopy has already gained strength to provide informative images of nanomaterials in living cells. Three major advantages of this new modality are: 1) to observe various label-free nanomaterials with specificity, coupled with the intrinsic properties of electronic states; 2) to monitor the nanomaterials with minimal spectral conflict from a strong fluorescence background, and; 3) to discriminate various nanomaterials by the lifetimes of their excited states, as transient absorption microscopy is time-resolved. A summary of nanomaterials studied by transient absorption is presented in Table 1. Although only carbon nanomaterials and gold nanorods have been clearly demonstrated in living cells or animals, other nanomaterials, including graphene, carbon dots, quantum dots, and others, will also fit this ‘universal’ microscopic bioimaging modality. We believe transient absorption microscopy will become a powerful tool to complement other nonlinear optical imaging technologies to visualize the complex interactions in bio-nanotechnology.

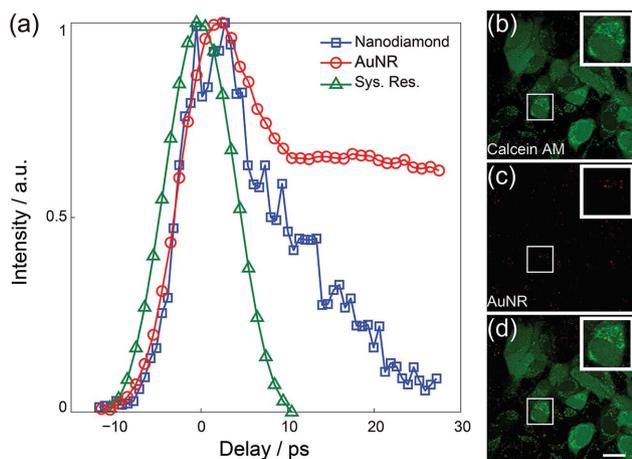


Figure 4. Imaging gold nanorods (AuNRs) in living cells with transient absorption microscopy. a) Different dynamics of transient absorption signals of nanodiamonds and AuNRs. The zero delay is determined by stimulated Raman scattering signal of paraffin. (Sys. Res.: systematic response.) b) Two-photon excited fluorescence of living cells labeled with calcein AM. c) Transient absorption image of AuNRs in cells. d) An overlay of calcein AM and AuNRs in living cells. All scale bars are 20 μm. Panels (b–d) adapted with permission.^[34] Copyright 2014, The Royal Society of Chemistry.

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