Published in final edited form as:

Nat Photonics. 2011 February ; 5(2): 110–116. doi:10.1038/nphoton.2010.280.

Ultrasharp nonlinear photothermal and photoacoustic resonances and holes beyond the spectral limit

Vladimir P. Zharov

Phillips Classic Laser and Nanomedicine Laboratories, University of Arkansas for Medical Sciences, 4301 W. Markham St., Little Rock, Arkansas 72205

Abstract

High-resolution nonlinear laser spectroscopy based on absorption saturation, Lamb-dip and spectral hole-burning phenomena have contributed much to basic and applied photonics. Here, a laser spectroscopy based on nonlinear photothermal and photoacoustic phenomena is presented. It shows ultrasharp resonances and dips up to a few nanometres wide in broad plasmonic spectra of nanoparticles. It also demonstrates narrowing of absorption spectra of dyes and chromophores, as well as an increase in the sensitivity and resolution of the spectral hole-burning technique. This approach can permit the study of laser-nanoparticle interactions at a level of resolution beyond the spectral limits, identification of weakly absorbing spectral holes, spectral optimization of photothermal nanotherapy, measurements of tiny red and blue plasmon resonance shifts, multispectral imaging and multicolour cytometry.

The unique optical properties of laser radiation, including its high intensity, monochromaticity and spectral tuning, have revolutionized spectroscopy by allowing the spectral resolution to be determined, not by an instrument's limits, but by natural spectral-line broadening^{1–4}. Nonlinear saturated absorption spectroscopy exploring Lamb-dip effects has enabled study of the ultrafine structures of spectral lines that is not limited by thermal motion of the molecules². Spectral hole-burning technique using phototransformation of molecules under a narrow-band resonant laser is a powerful tool for studying tiny structures in multiple inhomogeneously broadened absorption bands^{5–7}. Carefully engineered nanoparticles with enhanced absorption, fluorescence and scattering properties has also enabled further progress in spectral imaging and detection of analytes^{8–13}. For example, quantum dots with narrow (~30 nm), tunable fluorescent peaks in the visible spectral range have resulted in a major improvement in multicolour (e.g., 5 colours) imaging, although broader peaks (60 to 90 nm) in the near-infrared window of transparency of biological tissue

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

Corresponding author's information: Vladimir Zharov (zharovvladimir@uams.edu).

Author Contribution. V.P.Z. proposed the concept of nonlinear PT and PA spectroscopy in 2005, performed its experimental verifications with the help of colleagues in 2005–2009, and wrote the paper.

Competing financial interests Statement

The author declares that he has no competing financial interests.

Additional information

Supplementary information accompanying this paper can be found at www.nature.com/naturephotonics

Reprint and permission information is available online at <http://npg.nature.com/reprintsandpermission/>.

(650 to 1100 nm) may limit their multicolour capacity¹⁰. Noble metal nanoparticles with tunable plasmon scattering resonances open alternative paths to multicolor imaging^{8,11–13}.

Photothermal (PT) and, especially, photoacoustic (PA) techniques based on nonradiative conversion of absorbed light energy into heat and accompanying acoustic effects have improved multispectral imaging by providing better resolution and bigger penetration depth compared with other optical modalities^{14–25}. PA and PT methods have been effective for studying weakly absorbing media, multiphoton and Raman spectroscopy, microscopy beyond the diffraction limit, PA tweezers, and *in vivo* detection of circulating tumour cells^{8,33–35,27–32}. Plasmonic gold nanoparticles have demonstrated their potential as PT/PA molecular contrast agents because of their ultrahigh near-infrared absorption, which is much greater than that of conventional dyes. They convert of absorbed energy into heat, acoustic waves, and nanobubbles effectively and swiftly, and their plasmon resonances can be tuned to a desired spectral range by varying the nanoparticle size, shape, and composition^{36–41}. However, because of their relatively broad plasmonic bands of 80 to 200 nm, the multiplexing capacity of these nanoparticles to target simultaneously several disease-associated markers is typically limited to two distinct nonoverlapping colours^{34,35}. The nonlinear spectroscopy described in this paper can resolve this problem by a dramatic sharpening of the absorption spectra of PT and PA contrast agents.

PRINCIPLES OF NONLINEAR PHOTOTHERMAL AND PHOTOACOUSTIC SPECTROSCOPY

In biological applications, a short laser pulse interacts initially with individual spectrally and spatially heterogeneous absorbing endogenous biomolecules (e.g., hemoglobin, melanin, or cytochromes) or exogenous dyes or nanoparticles (Fig. 1a). Most of these absorbers having typically nanoscale sizes may form nano- and microclusters in various biological environments such as cytochromes in mitochondria, melanin in melanosomes, dye molecular assembly, and synthetic nanoparticles around dense biomarkers.^{20,33,40} Because of the close proximity of these nanoabsorbers in clusters, their laser-induced optical, thermal, acoustic and nanobubble phenomena may overlap spatially and temporally, causing nonlinear enhancement of PT and PA effects⁴⁰. With increases in the laser energy level, the temperatures of the nanoabsorbers quickly reach the thresholds of phase effects, such as evaporation of liquid surrounding heated nanoparticles (250 to 350 °C for water), nanoparticle melting (1,063°C for gold), and nanoparticle evaporation (2,710°C for gold)^{42–45}. Starting at the picosecond temporal scale due to fast nonradiative relaxation, temperature-dependent changes of gold nanoparticle (e.g., nanorods or nanoshells) shape or size during the laser pulses lead to blue spectral shifts as absorption is depleted in the centre of near-infrared resonances and longer wavelengths, and increased at shorter wavelengths^{42–45}. Thus, the total laser energy deposition in the nanoparticles can be lower or higher for resonant and red-shifted or nonresonant, blue-shifted laser radiation, respectively. A nonlinear change in energy deposition, can occur at the same laser wavelength and different energy levels (see Supplementary Information; Fig. S1).

As we previously observed using a multimodal set-up,^{27,33–34,39–40} PA and PT signals from nanoparticles, dyes and cells exhibits similar multistage behaviors when the energy fluence

(E) was increased (Fig. S2, S3; Supplementary Information). These behaviours, summarized in Fig. 1b, include (1) a gradual linear increase at a low fluence, E^n ($n = 1$); (2) strong (10–100-fold) nonlinear signal amplification ($n > 1$); and (3) signal complete or slight saturation ($n \sim 0$ to 0.5). At higher energy, signals can be further enhanced or inhibited depending on nanoparticle type and dominant nonlinear effects (e.g., nanoparticle explosion). Signal amplification in stage (2) is associated with nano- and microbubble formation with a well-defined threshold, E_{TH} . It occurs in nanoparticles at lower thresholds, a narrower energy fluence range, $E/E_{TH} \sim 0.2$ to 0.6, and with higher nonlinearity ($n \sim 3$ to 5) than in other samples ($E/E_{TH} \sim 0.8$ to 1.2; $n = 1.5$ to 2). In some cases, we also observed small signal inhibition in the vicinity of the thresholds only. The offset of a signal plateau is likely related to saturation of size, velocity of growth and overlapping of bubbles, or phase transitions (e.g., evaporation or melting) occurring at a constant temperature^{27,30,33–34}.

In view of the described phenomena, the principles of nonlinear PT-based spectroscopy can be presented as follows. At a given laser energy level close to the threshold E_{TH} , nonlinear PT and PA signals appear most prominently in the centres of absorption bands, where the relationship between signal amplitude and laser energy shows stronger nonlinearity ($\sim E^n$; $n \sim 1.5$ to 5). If the laser wavelength shifts away from the absorption centres and the laser-induced temperature is slightly below the threshold for nonlinear effects (e.g., bubble formation), a linear signal is generated. However, a shift of the laser wavelength toward the absorption centres leads to an increase in energy absorption, and hence in the temperature of the absorbing zone. A temperature slightly above the evaporation threshold induces sudden nanobubble formation accompanied by nonlinear signal amplification. As a result, spectrally dependent signal amplification will lead to the sharpening of PT/PA resonances near the centre of the absorption peak (Fig. 1c).

Nonlinear PT and PA phenomena may also increase both the sensitivity and spectral resolution of the spectral hole-burning technique for identifying individual nanoparticles in a mixture of weakly absorbing nanoparticles with overlapping spectra. Laser radiation can burn and thus deplete only nanoparticles whose absorption peak is precisely resonant with the laser energy (Fig. 1d). At a high energy level far above the threshold of nonlinear effects, a laser will burn a broad spectral hole and form one blue-shifted PT/PA resonance or two red- and blue-shifted resonances (Fig. 1e), depending on the absorber types and the laser energy levels. Time-resolved pump–probe excitation technique (Fig. 1a) employing a laser energy level close to the nonlinear threshold may permit observation of narrower spectral holes in the centres of homogeneously broadened nanoparticle bands (Fig. 1c, blue curve).

RESULTS

Ultrasharp resonances and holes in gold nanorods

This phenomenological model was first verified with the use of gold nanorods whose longitudinal plasmon resonances could be adjusted in the spectral range of 520 to 1200 nm^{37–38}. Nonlinear PT spectra of the nanorods were obtained by measuring the amplitude of the negative PT peaks at different laser wavelengths but the same pulse energy level. Laser spectral scanning was combined with spatial scanning by automatic movement of the microscopic stage to avoid cumulative effects. For nanorods with an absorption maximum at

550 nm, the 8.8-nm-wide peak that we obtained in the PT spectrum at an amplitude level 70% of maximum was 6.3-fold narrower than the 55-nm-wide peak in the absorption spectra for the same nanorods (Fig. 2a). PT holes were observed with the pump–probe excitation technique as a function of spectral detuning of the pump pulse at a fixed probe wavelength of 639 nm and an energy fluence of the probe pulse of 1 J/cm². At a low pump laser energy level, the spectral width of the hole was relatively narrow, ~9 nm (Fig. 2b), while a higher pump laser energy level led to broadening, blue shifting, and asymmetry of the hole. Latter effects were associated with laser-induced modification of the shape of nanorods mentioned above that led to more profound absorption depletion at longer wavelengths and absorption enhancement at shorter wavelengths^{42–45}. Similar phenomena were also observed in one-beam excitation mode, when the central resonances disappeared with an increase in the energy level, and two PT resonances formed instead, with red and blue shifts and asymmetry that were more profound at the high laser energy level with dominant blue-shifted resonances (Fig. 2c). Simultaneous measurement of PT and PA spectra near the centre of the absorption peak showed similar results: an approximately 10-fold their sharpening at a laser energy level close to the nonlinear threshold (Fig. 2c, red curves). Ultrasharp PT resonances made it possible to easily identify each nanorod in a mixture of six nanorods with nearly overlapping plasmon resonances that were hardly distinguishable in conventional absorption spectra (Fig. 2d).

In a spectral hole-burning experiment, the measurements consisted of three steps: (1) a linear signal baseline in the absorption band of nanorods was generated by pump laser scanning at a low pulse energy fluence of 45 mJ/cm² over the wavelength range of interest, 500 to 1100 nm; (2) two ruby laser pulses at a wavelength of 690 nm and a fluence of 0.3 J/cm² were used to burn the hole; and (3) the hole was probed by laser scanning as in the first step. Linear PT/PA spectra at a low laser energy level revealed the high contrast of the spectral hole in the 120-μm-thick nanoparticle solution, which was almost invisible with absorption spectroscopy (Fig. 2e). The observed hole's width of approximately 95 nm was determined by a conventional homogeneous band of nanorods with plasmon resonances at approximately 717 nm (destroyed by the burning laser) in the inhomogeneous linear absorption spectra of a mixture of seven nanoparticles. Nonlinear PT/PA spectra of the same mixture near 717 nm showed narrowing of the spectral hole to ~28 nm due to signal enhancement out the centre of the hole at a laser energy level close to the threshold in this centre. For comparison, a two-beam PT technique provided a narrower hole width of 4.4 nm in an absorption band of 89-nm-wide nanorods (Fig. 2b).

Ultrasharp resonances in quantum dots and gold-coated nanoparticles

The study of ultrasharp resonances was extended to golden carbon nanotubes³⁹, quantum dots^{9–11}, and gold nanoshells³⁶. The sharpening, to approximately 7 nm, was found for the longitudinal plasmon resonance of golden carbon nanotubes (Fig. 3a). The nonlinear PT spectra of quantum dots exhibited two resonances near absorption peaks at 535 nm and 560 nm, and one resonance at 491 nm outside the profound absorption peaks. The width of resonance at 560 nm was approximately 6-nm, i.e., 5-fold narrower than the emission spectra of quantum dots (Fig. 3b), suggesting that nonlinear PA spectroscopy provides a better spectral capacity than fluorescence spectroscopy for quantum dots. The origin of the

resonance near 491 nm is explained by bubble-induced amplification of PT signals as the laser wavelength decreased with further signal inhibition due to nanoparticle modification decreasing energy deposition. The PA resonances of gold nanoshells, whose absorption bands were considerably broader than those of gold nanorods (150 to 350 nm vs. 60 to 100 nm), were likewise broader: 35 to 100 nm vs. 8 to 20 nm (Fig. 3c vs. Fig. 2d).

Narrow resonances in cellular and bacterial chromophores

Next, this new method of laser spectroscopy was applied to cells and bacteria. Measurements of the PT spectra of individual mitochondria in KB-3 human carcinoma cells with a focused 1- μ m-diameter laser beam showed good correlation with the absorption spectrum of cytochrome *c* as the main absorbing component in mitochondria in the visible spectral range⁴⁶. However, the PT peak was narrower due to nonlinear sharpening effects (Fig. 4a, right).

Absorption spectra of mouse blood containing B16F10 melanoma cells revealed that the strong background absorption of blood masked the weak absorption from melanoma cells. Nonlinear PT spectroscopy of the same sample at an energy fluence of 0.7 J/cm² showed narrow resonance peaks from the melanoma cells emerging from the blood background near 500 nm (Fig. 4b). This increase in the spectral contrast of melanoma cells was associated with nonlinear amplification of signals from melanin nanoclusters in the melanoma cells³³, which have a small absorption peak at 500 nm in the absence of notable nonlinear effects at the laser energy used in blood with smoother absorption spectra near this wavelength. The sharpening of PT spectra of blood occurred at 550–590 nm (Fig. 4b). Thus, nonlinear PT/PA spectroscopy may detect weak absorption peaks masked by strong background signals with smooth absorption spectra. Similar spectrum-sharpening phenomena were observed in *Staphylococcus aureus*, which were associated with absorption of endogenous carotenoids with two specific peaks near 485 nm and 740 nm (Fig. 4c).

Narrowing of absorption peaks of conventional dyes

Finally, nonlinear effects were studied in the conventional dyes such as indocyanine green and fluorescein isothiocyanate (FITC) (Fig. 5). With increased laser energy at a wavelength coinciding with the maximum absorption of these dyes, PA signals exhibited nonlinear behaviors similar to, but less profound than, those of nanoparticles (Fig. S3; Supplementary Information). The absorption band width of indocyanine green at 780 nm narrowed from 160 nm to 70 nm at a laser fluence of 0.5 J/cm². When FITC was used to label a small amount of mouse erythrocytes, nonlinear effects in these cells made it possible to selectively detect them against the strong background absorption from non-labeled cells (Fig. 5b). The nonlinear PA absorption peak was approximately three times narrower than the linear absorption peak of FITC. The observed nonlinear PT and PA phenomena are likely enhanced by the presence of nano- and microscale molecular aggregates in the dye solutions²⁰.

DISCUSSION

The spectral sharpening effects were found to be dependent on the initial width of the absorption profile (the narrower, the better), degree of nonlinearity (the higher, the better), distribution of target sizes and local absorption (the more homogenous, the sharper), and the stability of laser energy during spectral scanning (the more stable, the narrower). The sharpening effects can make the absorption lines of the plasmonic nanoparticles, photonic crystals, metamaterials and conventional dyes narrower independently of the initial narrowness of the line and the physical phenomena limiting their width and line shape (e.g., symmetric-Lorentzian or asymmetric-Fano), in particular, interference, plasmon resonance, or surface enhanced resonance scattering (SERS).^{36–38,47} As nonlinear PT/PA signal amplification can occur at centres of any absorption lines, even supernarrow, the width of nonlinear PA/PT resonances can be beyond the conventional spectral limits. The best result of an approximately 10-fold sharpening to 4 to 9 nm was achieved for gold nanorods and golden carbon nanotubes at a laser energy stability of 1.8%. For more heterogeneous absorbing structures with relatively broad absorption bands, such as dyes and cell chromophores, sharpening was less profound (2- to 3-fold), with a final linewidth of 25 to 30 nm. According to the Rayleigh criterion, for gold nanorods with 10-nm-wide resonance, up to 40 nonoverlapping, ultrasharp PA resonances can be distinguished in the near-infrared range, although 15–20 colours seem to be more realistic numbers. Nevertheless, this number of colours is much higher than that for fluorescent quantum dots in the near-infrared range.

The nonlinear PT-based laser spectroscopy described here is relatively universal and with slight modifications it can be used in various PT schematics such as infrared radiometry, phase- and interference-contrast microscopy, measurements of elastic and Raman scattering or fluorescence and sonoluminescence from bubbles, SERS particles, or plasma- and cavitation-induced radicals^{20–31,48}. The refraction and scattering effects on bubbles during laser excitation can decrease laser energy deposition in nanoparticles thus contributing to sharpening effects (Fig. S4; Supplementary Information).

The nonlinear pump–probe techniques used here to observe PT/PA resonances and dips are similar to nonlinear saturation spectroscopy exploring Bennet peaks and Lamb dips⁴. However, the physical mechanisms underlying nonlinear PA and PT spectroscopy are different. They are related to high-order nonlinear processes responsible for PA and PT effects, which may include bubble generation, multiphoton absorption and ionization, thermal explosion, evaporation, melting and shock wave formation^{3,20,27,30,42–45}. Nonlinear sharpening of spectral lines near absorption maxima is roughly analogous to laser function, which is based on nonlinear light amplification near the centre of the spectral line of active medium accompanied by dramatic narrowing of the spectral width of the amplified light^{1,4}. The high sensitivity of PA and PT spectroscopy^{3,20–22} makes it possible to apply the spectral hole-burning technique to the study of weakly absorbing, nonfluorescent, inhomogeneously broadened nanoparticles at a low concentration, which is impossible with conventional methods^{5–7}. Moreover, a pump–probe mode also allows the observation of spectral holes in homogeneously broadened spectral lines of gold nanoparticles (Fig. 2b).

The high sensitivity of the PT/PA technique allows us to explore highly localized nonlinear phenomena near the threshold of their generation when they could not be harmful to surrounding tissue. The laser fluence required is close to the safety standard of 35 to 100 mJ/cm² for 700- to 1100-nm for humans^{20–22}. It can be further reduced by using strongly absorbing nanoparticles with a threshold for nonlinear effects at approximately 5 mJ/cm^{38,42,45} and use nanosecond lasers, that are more appropriate for spectroscopic applications because of the inherently large spectral bandwidths of picosecond and femtosecond laser pulses⁴.

In conclusion, nonlinear ultrasharp PT/PA spectral resonances accompanied by amplification of PT/PA effects may lead to dramatic increases in both the specificity and sensitivity of PT/PA spectral analysis and the efficiency of PT therapy. Potential applications include identifications of nanoparticles and cells masked by background absorption of biological tissue or blood, study of the behaviours of nanoscale systems in a strong laser field, multicolour cytometry, multispectral imaging, detection of multiple disease-associated biomarkers, accurate measurements of tiny red and blue shifts in plasmonic nanoparticle clusters and multiplex PT therapy. The precise matching of resonant laser wavelengths with absorption centres and optimal laser energy is required to generate nonlinear effects in these centres to provide highly spectral selective diagnosis and therapy. At higher laser energy above optimal levels, energy-dependent spectral shifts, especially the blue shift in PT/PA resonances should be taken into account to achieve maximum diagnostic and therapeutic effects.

METHODS

Integrated PA and PT microscopes

Laser-induced PT and accompanying PA and bubble-formation phenomena in samples were evaluated with an integrated PA/PT microscope described in detail elsewhere^{20,39–40}. Briefly, the first set-up (Fig. S2a; Supplementary Information) was built on the technical platform of an upright Olympus BX51 microscope (Olympus America, Inc.) onto which PA, PT, fluorescent, and transmission modules were incorporated. A tunable optical parametric oscillator (OPO)-laser (Lotis Ltd., Minsk, Belarus) was used to irradiate samples in 120- μ m-thick microscope slides at the following parameters: wavelength, 420 to 2,300 nm; pulse width, 8 ns; beam diameter, 10 to 50 μ m; fluence range, 1 to 10⁴ mJ/cm²; and pulse repetition rate, 10 Hz. Laser-induced PA waves were detected with an ultrasound transducer (XMS-310, 10 MHz; Panametrics) that was gently attached at the slide. Warm water or ultrasound gel was applied for better acoustic matching between the transducer and the samples. After amplification (amplifier model #5662, bandwidth, 50 kHz to 5 MHz; gain, 60 dB; Panametrics), signals were recorded by either a computer or a Tektronix TDS 3032B oscilloscope, and then analysed with standard and customized software. The PA signals had a bipolar shape that was transformed into a pulse train due to reflection and resonance effects (Fig. 1a, bottom). The set-up was equipped with a high-speed (200 MHz) analog-to-digital converter board (National Instruments Corp., PCI-5152, 12-bit card, 128 MB of memory), specialized software (LabVIEW; National Instruments), and a Dell Precision 690

workstation with a quadcore processor, 4 GB of RAM, and Windows Vista 64-bit operating system.

In PT thermolens schematic, laser-induced temperature-dependent variations of the refractive index around absorbing zones in a liquid environment caused defocusing of a continuous-wave collinear He-Ne laser (model 117A, Spectra-Physics, Inc.; wavelength, 633 nm; 1.4 mW) probe beam, leading to a subsequent reduction in the beam's intensity at its centre, detected by a photodiode (C5658; Hamamatsu Corp.). The linear PT thermolens signals demonstrated the standard fast-rising unipolar peak associated with rapid (picosecond-nanosecond scale) sample heating and a slower (microsecond scale) tail corresponding to sample cooling (Fig. 1a, right, top). At a high laser energy level, the local temperature around strongly absorbing zones reaches the bubble-formation threshold, leading to the appearance of a negative peak in the nonlinear PT signals (Fig. 1a, right, bottom) due to bubble-induced local refraction and scattering effects. To provide PT imaging, the refractive-index variations were visualized with multiplex thermolens schematics and a CCD camera (Apogee), using a pump (OPO) and a second laser probe pulse (Raman shifter; wavelength, 639 nm; pulse width, 12 ns; pulse energy, 2 nJ; delay, 0 to 10 μ s).⁴⁵ This pump-probe schematic was also used for two-beam excitation of gold nanoparticles.

The second set-up was built on the technical platform of an Olympus IX81 inverted microscope (Olympus America, Inc.) (Fig. S2b) with the use of a tunable OPO (Opotette HR 355 LD, Opotek, Inc.) with a 5-ns wide laser pulse, a repetition rate up to 100 Hz, a wavelength range of 410 to 2,500 nm, and a fluence range of 1 to 10⁴ mJ/cm². PT/PA mapping of the sample was realized by sample-scanning with two-dimensional (X-Y) translation stage (H117 ProScan II, Prior Scientific, Inc.) with a positioning accuracy of 50 nm and laser spot size of approximately 1 μ m at 20 $\times$ magnification in near-infrared range. Focusing along the Z-axis was performed by moving the microscopic objective axially. PT thermolens signal detection was similar to the first set-up. Synchronization of the excitation laser, signal acquisition/processing, and translation-stage control were implemented in a single software module (in-house software based on LabView 8.5 complex, National Instruments, Inc.).

Nanoparticles and dyes

Various sizes of gold nanorods having maximum absorption at 550 nm, 600 nm, 650 nm, 700 nm, 717 nm, 750 nm, 780 nm, 808 nm, 878 nm, and 1064 nm were provided by Nanopartz, Inc. The 30-nm gold nanospheres with absorption at ~525 nm were obtained from Ted Pella, Inc. The initial study was performed with 15–50-nm gold nanorods whose maximum absorption was near 850 nm and silica/gold nanoshells (140–15 nm) whose maximum absorption was near 900 nm. The nanoshells were provided by the laboratory of Nanoscale Biosensors at the Institute of Biochemistry and Physiology of Plants and Microorganisms (Saratov, Russia). Golden carbon nanotubes averaging 12–98 nm in size were provided by Dr. Kim³⁹. Indocyanine green and FITC were purchased from Akorn, Inc. The optical absorption spectra of nanoparticles and dyes were examined at the room temperature by the DU 800 ultraviolet/visible/ near infrared spectrophotometer (Beckman

Coulter, Inc.) and an optical cuvette with quartz windows and dimension of 2×10×10 mm (light path, 10 mm; suspension height, 2–8 mm).

Cells

All cancer cell lines were from the American Type Culture Collection (ATCC), Rockville, MD. KB-3 human carcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 U/mL penicillin, 50 µg/mL streptomycin, and 5 mM L-glutamine at 37°C and 5% CO₂. B16F10 mouse melanoma cells were cultured according to standard procedures, including serial passage in phenol-free RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). The *S. aureus* strain-designated UAMS-1 was isolated from a patient with osteomyelitis at the McClellan Veterans Hospital in Little Rock, Arkansas, USA. UAMS-1 was cultured in tryptic soy broth and grown aerobically for 16 h at 37 °C. Cells were harvested by centrifugation and resuspended in sterile phosphate-buffered saline. Fresh blood was obtained from mice, collected in heparinized tubes, and spiked with different numbers of B16F10 mouse melanoma cells.

Statistical methods

Results are expressed as means plus/minus the standard error of at least three independent experiments ($p < 0.05$). Statistica 5.11 (StatSoft, Inc., Tulsa, OK) and MATLAB 7.0.1 (MathWorks) were used for the statistical calculations. Data were summarized as the mean, standard deviation (SD), median, interquartile range, and full range. Spearman correlations for which $p < 0.05$ were considered as statistically significant. Error bars, typically in the range of 15% to 25% for each wavelength or laser energy level, represent the standard deviation of five measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health (R01EB000873, R01CA131164, R01EB009230, R21EB0005123, and R21CA139373) and the National Science Foundation (DBI-0852737). I would like to thank J.-W. Kim for providing golden carbon nanotubes; N. Khlebtsov for providing gold nanorods and nanoshells; D. Lapotko for help with building the first (Fig. S2a) PT microscope; E.V. Shashkov, D.A. Nedosekin, and S. Fergusson for their assistance with laser measurements; Jian-Hui Ye for sample preparation, and E.I. Galanzha, M. Sarimollaoglu, and W. Gabello for help with preparation of the manuscript.

References

1. Lamb WE. Theory of an optical maser. Phys Rev. 1964; 134:A1429–A1450.
2. Letokhov VS. Nonlinear high resolution laser spectroscopy. Science. 1975; 190:344–351. [PubMed: 1179214]
3. Zharov, VP.; Letokhov, VS. Laser optoacoustic spectroscopy. Springer-Verlag; New York Springer: 1986.
4. Demtröder, W. *Laser spectroscopy series: Advanced Texts in Physics*. XVI. Berlin Heidelberg: 2008. p. 4

5. Moerner, WE.; Bjorklund, GC. Persistent spectral hole-burning: science and applications. Springer-Verlag; Heidelberg, New York: 1988.
6. Stietz F, et al. Decay times of surface Plasmon excitation in metal nanoparticles by persistent spectral hole burning. *Phys Rev Letters*. 2000; 24:5644–5647.
7. Kazakevich PV, Simakin AV, Shafeev GA. Laser burning of a gap in the spectrum of plasmon resonance of gold nanoparticles. *Chemical Physics Letters*. 2006; 421:348–350.
8. Giljohann DA, Mirkin CA. Drivers of biodiagnostic development. *Nature*. 2009; 462:461–464. [PubMed: 19940916]
9. Larson DR, et al. Water-soluble quantum dots for multiphoton fluorescence imaging in vivo. *Science*. 2003; 300:1434–1436. [PubMed: 12775841]
10. Kobayashi H, et al. Simultaneous multicolor imaging of five different lymphatic basins using quantum dots. *Nano Lett*. 2007; 7:1711–1716. [PubMed: 17530812]
11. Vo-Dinh T, Wang HN, Scaffidi J. Plasmonic nanoprobe for SERS biosensing and bioimaging. *J Biophotonics*. 2010; 3:89–102. [PubMed: 19517422]
12. Chen K, Liu Y, Ameer G, Backman V. Optimal design of structured nanospheres for ultrasharp light-scattering resonances as molecular imaging multilabels. *J Biomedical Optics*. 2005; 10:024005. [PubMed: 15910079]
13. Khlebtsov B, Khlebtsov N. Ultrasharp light-scattering resonances of structured nanospheres: effects of size-dependent dielectric functions. *J Biomedical Optics*. 2006; 11:044002. [PubMed: 16965159]
14. Bell AG. On the production and reproduction of sound by light. *Am J Sci*. 1880; 20:305–324.
15. Boccara AC, Fournier D, Badoz J. Thermo-optical spectroscopy: detection by the mirage effect. *Appl Phys Lett*. 1980; 36:130–132.
16. Zharov VP, Montanari SG. Laser optothermal detection for capillary chromatography. *Laser Chem*. 1985; 5:133–142.
17. Shibata MM, Kitamori T, Sawada T. Application of coaxial beam photothermal microscopy to analysis of a single biological cell in water. *Anal Chim Acta*. 1995; 299:343–347.
18. Lapotko D, Kuchinsky G, Potapnev M, Pechkovsky D. Photothermal image cytometry of human neutrophils. *Cytometry*. 1996; 24:198–203. [PubMed: 8800552]
19. Boyer D, et al. Photothermal imaging of nanometer-sized metal particles among scatterers. *Science*. 2002; 297:1160–1163. [PubMed: 12183624]
20. Zharov VP, Lapotko DO. Photothermal imaging of nanoparticles and cells. *IEEE J Sel Topics Quant Electron*. 2005; 11:733–751.
21. Tanaka Y, et al. Biological cells on microchips: new technologies and applications. *Biosens Bioelectron*. 2007; 23:449–458. [PubMed: 17881213]
22. Cognet L, Berciaud S, Lasne D, Lounis B. Photothermal methods for single nonluminescent nano-objects. *Anal Chem*. 2008; 80:2288–2294. [PubMed: 18456908]
23. Wang LV. Multiscale photoacoustic microscopy and computed tomography. *Nature Photon*. 2009; 3:503–509.
24. Mallidi S, et al. Multiwavelength photoacoustic imaging and plasma resonance coupling of gold nanoparticles for selective detection of cancer. *Nano Lett*. 2009; 9:2825–2831. [PubMed: 19572747]
25. Rasansky D, et al. Multispectral opto-acoustic tomography of deep-seated fluorescent proteins in vivo. *Nature Photon*. 2009; 3:412–417.
26. Zharov V. Far-field photothermal microscopy beyond the diffraction limit. *Opt Lett*. 2003; 28:1314–1316. [PubMed: 12906074]
27. Beilin E, et al. Investigation of laser-induced acoustic effects in water and their influence on cell structure. *Akustichesky Zhurnal (Soviet Physics Acoustics)*. 1987; 33:344–349.
28. Zharov VP, Latyshev AS. Laser combined medical technologies from Russia. *J Laser Application*. 1999; 11:80–90.
29. Diebold GJ, Khan MI, Park SM. Photoacoustic “signatures” of particulate matter: optical production of acoustic monopole radiation. *Science*. 1990; 250:101–104. [PubMed: 17808241]

30. Egerev S, et al. Acoustic signals generated by laser-irradiated metal nanoparticles. *Appl Opt.* 2009; 48:38–45.
31. Zharov V, Galitovsky V, Viegas M. Photothermal detection of local thermal effects during selective nanophotothermolysis. *Appl Phys Lett.* 2003; 83:4897–4899.
32. Zharov V, Malinsky T, Richard K. Photoacoustic tweezers with a pulsed laser: theory and experiments. *J Physics D: Appl Phys.* 2005; 38:2662–2674.
33. Galanzha EI, Shashkov EV, Spring P, Suen JY, Zharov VP. *In vivo* noninvasive label-free detection and eradication of circulating metastatic melanoma cells by two-color photoacoustic flow cytometry and a diode laser. *Cancer Research.* 2009; 69:7926–7934. [PubMed: 19826056]
34. Galanzha EI, et al. *In vivo* magnetic enrichment and multiplex photoacoustic detection of circulating tumour cells. *Nature of Nanotechnol.* 2009; 12:855–860. [PubMed: 19915570]
35. Li PC, et al. Photoacoustic imaging of multiple targets using gold nanorods. *IEEE Trans Ultrason Ferroelectr Freq Control.* 2007; 54:1642–1647. [PubMed: 17703668]
36. Hirsch LR, et al. Metal nanoshells. *Ann Biomed Eng.* 2006; 34:15–22. [PubMed: 16528617]
37. Huang X, Jain PK, El-Sayed IH, El-Sayed MA. Plasmonic photothermal therapy (PPTT) using gold nanoparticles. *Lasers Med Sci.* 2008; 23:217–228. [PubMed: 17674122]
38. Pissuwan D, Valenzuela SM, Cortie MB. Therapeutic possibilities of plasmonically heated gold nanoparticles. *Trends Biotechnol.* 2006; 24:62–67. [PubMed: 16380179]
39. Kim J-W, Galanzha EI, Shashkov EV, Moon H-M, Zharov VP. Golden carbon nanotubes as multimodal photoacoustic and photothermal high-contrast molecular agents. *Nature of Nanotechnol.* 2009; 4:688–694. [PubMed: 19809462]
40. Zharov VP, Kim JW, Everts M, Curiel DT. Self-assembling nanoclusters in living systems: application for integrated photothermal nanodiagnostics and therapy. *J Nanomedicine.* 2005; 1:326–345.
41. Yang X, Stein EW, Ashkenazi S, Wang LV. Nanoparticles for photoacoustic imaging. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2009; 1:60–368. [PubMed: 20049779]
42. Aguirre CM, Moran CE, Young JF, Halas NJ. Laser induced reshaping of metallodielectric nanoshells under femtosecond and nanosecond plasmon resonant illumination. *J Phys Chem B.* 2004; 108:7040–7045.
43. Chang S-S, et al. The shape transition of gold nanorods. *Langmuir.* 1999; 15:701–709.
44. Link S, Burda C, Nikoobakht B, El-Sayed MA. Laser-induced shape changes of colloidal gold nanorods using femtosecond and nanosecond laser pulses. *J Phys Chem B.* 2000; 104:6152–6163.
45. Akchurin G, et al. Gold nanoshell photomodification under single nanosecond laser pulse accompanied by color-shifting and bubble formation phenomena. *Nanotechnology.* 2008; 19:015701. [PubMed: 21730542]
46. Zharov V, Galitovskiy V, Lyle CS, Chambers TC. Super high-sensitive photothermal monitoring of individual cell response to antitumor drug. *J Biomed Opt.* 2006; 11:06403.
47. Luk'yanchuk B, et al. The Fano resonance in plasmonic nanostructures and metamaterials. *Nat Mater.* 2010; 9:707–715. [PubMed: 20733610]
48. Lee H, Alt C, Pitsillides CM, Lin CP. Optical detection of intracellular cavitation during selective laser targeting of the retinal pigment epithelium: dependence of cell death mechanism on pulse duration. *J Biomed Opt.* 2007; 12:064034. [PubMed: 18163850]

Figure 1. Phenomenologic model of nonlinear PA/PT spectroscopy

(a) General diagram. (b) Typical signal amplitude as a function of laser energy. (c) Ultrasharp PA/PT resonances and dips in a homogenous absorption profile. (d) PA guidance of spectral-hole burning in a mixture of gold nanoparticles with overlapping spectra. (e) Double PA/PT resonances with energy-dependent red and blue shifts and a central broad PA/PT hole.

Fig. 2. Nonlinear spectra of gold nanorods

(a) PT resonance in 10–25-nm gold nanorods at 550 nm. (b) PT dips obtained with the pump-probe technique in 10–35-nm gold nanorods with a plasmon resonance at 650 nm. (c) Nonlinear resonances obtained with a one-beam schematic in 15–45-nm gold nanorods with plasmon resonances at 860 nm. (d) Nonlinear PT spectra of a mixture of 30-nm gold nanospheres and six gold nanorods with resonances at 525, 585, 650, 700, 753, 870, and 1064 nm. (e) PT monitoring of spectral hole-burning in a mixture of six gold nanorods with plasmon resonances at 585, 650, 717, 775, 808, and 870 nm at energy laser fluence of 0.5 J/cm². Black line shows narrowing of the spectral hole at 2.5 J/cm². PA/PT signal amplitudes were normalized to the maximum linear absorption spectrum. The error bars represent the standard error of the mean.

Figure 3. Ultrasharp resonances in gold-based nanoparticles and quantum dots

(a) PA resonance (dashed red line) of 11.8–98-nm golden carbon nanotubes, normalized on the optical absorption spectra (solid black curve) at 800 nm. (b) Absorption spectra, emission spectra, and PA resonances of quantum dot-folate conjugates (0.5-hr incubation at 22°C) in MB-MDA-231 breast cancer cells, normalized on the maximum absorption of conventional spectra at the wavelength of 560 nm. (c) Spectra of 160-nm gold nanoshells. The error bars represent the standard error of the mean.

Figure 4. Sharpening of PT spectra of cells

(a) PT spectra of mitochondrion marked by a dashed red line in the PT image of cancer cell (the inset). (b) PT spectral identification of rare melanoma cells in mouse blood. The inset shows a single melanoma cell in blood. (c) PT and absorption spectra of *S. aureus* (the inset). Arrows indicate the positions of the maximum spectral absorption of carotenoids. The error bars represent the standard error of the mean.

Figure 5. PA and conventional absorption spectra of dyes

(a) Absorption spectra of an 11.5- μ L solution of indocyanine green and its PA spectra at different laser fluences. PT spectra were normalized on absorption spectra. (b) Absorption spectra of FITC alone (green). Absorption (blue) and nonlinear PA (red) spectra of FITC-labeled mouse erythrocytes. The error bars represent the standard error of the mean.