

Video Article

Measurement of Scattering Nonlinearities from a Single Plasmonic Nanoparticle

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Abstract

Plasmonics, which are based on the collective oscillation of electrons due to light excitation, involve strongly enhanced local electric fields and thus have potential applications in nonlinear optics, which requires extraordinary optical intensity. One of the most studied nonlinearities in plasmonics is nonlinear absorption, including saturation and reverse saturation behaviors. Although scattering and absorption in nanoparticles are closely correlated by the Mie theory, there has been no report of nonlinearities in plasmonic scattering until very recently.

Last year, not only saturation, but also reverse saturation of scattering in an isolated plasmonic particle was demonstrated for the first time. The results showed that saturable scattering exhibits clear wavelength dependence, which seems to be directly linked to the localized surface plasmon resonance (LSPR). Combined with the intensity-dependent measurements, the results suggest the possibility of a common mechanism underlying the nonlinear behaviors of scattering and absorption. These nonlinearities of scattering from a single gold nanosphere (GNS) are widely applicable, including in super-resolution microscopy and optical switches.

In this paper, it is described in detail how to measure nonlinearity of scattering in a single GNP and how to employ the super-resolution technique to enhance the optical imaging resolution based on saturable scattering. This discovery features the first super-resolution microscopy based on nonlinear scattering, which is a novel non-bleaching contrast method that can achieve a resolution as low as $\lambda/8$ and will potentially be useful in biomedicine and material studies.

Video Link

The video component of this article can be found at <https://www.jove.com/video/53338/>

Introduction

The study of plasmonics has attracted great interest due to its applications in many different fields¹⁻⁴. One of the most investigated fields in plasmonics is surface plasmonics, in which the collective oscillation of conduction electrons couples with an external electromagnetic wave at an interface between a metal and dielectric. Surface plasmonics has been explored for its potential applications in subwavelength optics, biophotonics, and microscopy^{5,6}. The strong field enhancement in the ultra-small volume of metallic nanoparticles due to localized surface plasmon resonance (LSPR) has attracted extensive attention, not only because of its exceptional sensitivity to particle sizes, particle shapes, and the dielectric properties of the surrounding medium⁷⁻¹⁰, but also because of its ability to boost inherently weak nonlinear optical effects¹¹. The exceptional sensitivity of LSPR is valuable for bio-sensing and near-field imaging techniques^{12,13}. On the other hand, the enhanced nonlinearity of plasmonic structures can be utilized in photonic integrated circuits in applications such as optical switching and all-optical signal processing^{14,15}. It is well known that the plasmonic absorption is linearly proportional to the excitation intensity at low intensity levels. When the excitation is strong enough, the absorption reaches saturation. Intriguingly, at higher intensities, the absorption increases again. These nonlinear effects are called saturable absorption (SA)¹⁵⁻¹⁷ and reverse saturable absorption (RSA)¹⁸, respectively.

It is known that due to the LSPR, scattering is particularly strong in plasmonic structures. Based on fundamental electromagnetics, the response of scattering versus incident intensity should be linear. However, in nanoparticles, scattering and absorption are closely linked via the Mie theory, and both can be expressed in terms of real and imaginary parts of the dielectric constant. Under the assumption that a single GNS behaves as a dipole under light illumination, the scattering coefficient (Q_{sca}) and absorption coefficient (Q_{abs}) from a single plasmonic nanoparticle according to the Mie theory can be expressed as¹⁹

$$Q_{\text{scat}} = \frac{8}{3} x^4 \left| \frac{m^2 - 1}{m^2 + 2} \right|^2 \text{ and } Q_{\text{abs}} = 4x \operatorname{Im} \left\{ \frac{m^2 - 1}{m^2 + 2} \right\}, \quad (1)$$

where x is $2\pi a/\lambda$, a is the radius of the sphere, and m^2 is ϵ_m/ϵ_d . Here, ϵ_m and ϵ_d correspond to the dielectric constants of the metal and of the surrounding dielectrics, respectively. Since the form of the scattering coefficient is similar to that of the absorption coefficient, it is therefore expected to observe saturable scattering in a single plasmonic nanoparticle²⁰.

Recently, nonlinear saturable scattering in an isolated plasmonic particle was demonstrated for the first time²¹. It is remarkable that at deep saturation, the scattering intensity in fact decreased slightly when the excitation intensity increased. Even more remarkably, when the excitation intensity continued increasing after the scattering became saturated, the scattering intensity rose again, showing the effect of reverse saturable scattering²⁰. Wavelength- and size-dependent studies have shown a strong relationship between LSPR and nonlinear scattering²¹. The intensity and wavelength dependences of plasmonic scattering are very similar to those of absorption, suggesting a common mechanism underlying these nonlinear behaviors.

In terms of applications, it is well known that nonlinearity helps to improve optical microscopy resolution. In 2007, saturated excitation (SAX) microscopy was proposed, which can enhance resolution by extracting the saturated signal via a temporal sinusoidal modulation of the excitation beam²². SAX microscopy is based on the concept that, for a laser focal spot, the intensity is stronger at the center than at the periphery. If the signal (either fluorescence or scattering) exhibits saturation behavior, the saturation must start from the center, while the linear response remains at the periphery. Therefore, if there is a method to extract only the saturated part, it will leave only the central part while rejecting the peripheral part, thus effectively enhancing the spatial resolution. In principle, there is no lower resolution limit in SAX microscopy, as long as deep saturation is reached and there is no sample damage due to the intense illumination.

It has been shown that the resolution of fluorescence imaging can be significantly enhanced by utilizing the SAX technique. However, fluorescence suffers from the photobleaching effect. Combining the discovery of scattering nonlinearity and the concept of SAX, super-resolution microscopy based on scattering can be realized²¹. Compared to conventional super-resolution microscopies, the scattering-based technique provides a novel non-bleaching contrast method. In this paper, a step-by-step description is given to outline the procedures required to obtain and extract the nonlinearity of plasmonic scattering. Methods of identifying scattering nonlinearities introduced by changing the incident intensity are described. More details will be provided to unravel how these nonlinearities affect images of single nanoparticles and how spatial resolution can be enhanced accordingly by the SAX technique.

Protocol

1. GNS Sample Preparation

1. Before preparing the sample, sonicate 1 ml GNS colloid solution for at least 15 min at about 40 kHz to prevent particle aggregation, which may cause the LSPR peak to shift.
2. Drop 100-200 μ l of GNS colloid onto a slide glass with commercial magnesium aluminum silicate (MAS) coating to fix the GNSs.
3. After at least 1 min, remove the extra colloid by flushing with distilled water. The waiting time depends on the required distribution density of the GNSs. Typically, 1-3 min results in a suitable density that enables the particles to be easily identified since most of them are isolated from each other. Significant aggregation may occur if the waiting time is too long.
4. Dry the sample by purging with nitrogen gas.
5. (Optional) To map the GNSs on the glass with high resolution, perform scanning electron microscopy (SEM) at this stage²³. An example image is provided in **Figure 1**, showing the characteristic density of GNSs. Use a field emission SEM to acquire the image. Once oil is added onto the sample (next step), it will be difficult to remove the oil and observe the sample with SEM.
6. Add a drop of oil with the same refractive index onto the sample to cover the GNSs and to eliminate the strong reflection from the glass substrate.
7. Place a cover glass on top of the sample and seal it with nail polish.
8. Wait at least 5 min until the nail polish dries. The sample is ready now.

2. Alignment of Home-built Confocal Microscope

1. See **Figure 2** for the scheme of setup. Align the white light illumination path of the microscope body itself. Turn on the halogen light source of the microscope, and follow the microscope manufacturer's manual to achieve the Köhler illumination condition. Ensure that the white halogen light beams are nearly parallel at the back of aperture of the objective, partially reflected by the 50/50 beamsplitter, and then propagate toward the laser.
2. Turn on the galvano mirrors to ensure that they remain at the correct initial position, that is, at the center of the scanning range.
3. Place at least two targets, made by a thin sheet of paper with concentric rings on it, along the halogen light path, and align them with the halogen beam.
4. To perform imaging, select the 532 nm laser. To perform spectroscopy measurements, select the super-continuum laser. During the alignment, the power of the lasers should be less than 10 μ W at the back aperture of the objective to avoid nonlinearity. Then, collimate the incident laser beam opposite to the outgoing halogen beam with the aid of the two targets. When this process is complete, the coarse alignment of the laser beam has been achieved.
5. Align the laser beam through the center of the back aperture of the objective lens. Typically, use an oil-immersion objective. Add a drop of oil between the oil-immersion objective lens and the GNS sample. Use a photomultiplier tube (PMT) as the detector to collect the scattering signals of the GNSs.

- Place a 20- μm -diameter pinhole in front of the PMT to block out-of-focus scattering signals. Turn on the galvano mirrors and PMT (via home-built software), adjust the pinhole position and height of the sample stage to maximize the backscattering signals of the GNSs, and then observe an individual GNS on a computer screen. A sample xy image of the GNSs with correct alignment is shown in **Figure 2B**.
- Slightly change the height of the sample stage to check the concentricity of the focus. If it is not concentric, adjust the beam with the two mirrors in front of the scanner until the center of the GNS remains at the same position while the height of the sample stage is changed. Make sure that the xz image of the PSF is similar to **Figure 2C** to ensure correct beam alignment. Process these two images with low-pass and Gaussian smooth filters.

3. Characterization of Scattering Nonlinearity

- At low excitation intensity (less than 10^4 W/cm^2), acquire an image of gold nanoparticles by following protocol 2.6.
- Open the image in ImageJ (or any other image analysis software). Draw a line across one of the GNSs in the image (see **Figure 2B**), and use Analysis -> Plot profile of the ImageJ tools to retrieve the scattering intensity profile. Fit the profile of the chosen PSF by a Gaussian function:

$$y = y_0 + \frac{A}{w\sqrt{\pi/2}} \exp\left[-2\frac{(x-x_c)^2}{w^2}\right], \quad (2)$$

where y is the PMT readout value, y_0 is the background value (if any), A is the peak amplitude, w is the width, x is the spatial coordinate, and x_c is the center coordinate of the Gaussian function. The FWHM of the corresponding PSF is $(\sqrt{\ln 2})w$. Based on the numerical aperture (NA) of the objective, the theoretical FWHM of the confocal PSF can be estimated to be approximately $0.43\lambda/\text{NA}$, where λ is the excitation wavelength. Compare these two numbers to check the alignment of the imaging system.

- Increase the excitation intensity by manually changing the neutral density (ND) filter in **Figure 2A**, and record the backscattering images at each intensity level. Take the value of the scattering signal from the center of each GNS at different excitation intensities, and plot the curve of scattering signals versus excitation intensities. Check the linearity of the first few points, which should exhibit a linear relationship when the excitation intensity is adequately low. Draw a line based on linear fitting of the first few points. If the scattering intensities of the subsequent points drop below this linear trend, saturation has occurred.
- After observing saturable scattering, gradually decrease the intensity below the saturation threshold, and image the same GNSs again to ensure the reversibility of the nonlinear behaviors.

4. Measurement of a Scattering Spectrum of a Single Gold Nanosphere

- To measure the backscattering spectrum from a single GNS, use the super-continuum laser as the laser source. The initial wavelength of the laser ranges from 450 nm to 1,750 nm. To remove the excess infrared power that might cause damage to the sample and the optical components, place one or two mirrors right after the super-continuum laser to reflect the visible light, and use beam dumps to collect the excess infrared light.
- Follow the alignment procedures in Section 2 to direct the super-continuum laser into the laser scanning confocal microscope. Use a broadband 50/50 BS to ensure spectral coverage across the whole visible range.
- Acquire an image of the GNSs on glass. Locate a single GNS in the image, and fix the focus of the incident broadband light on the particle.
- Use a flipping mirror in front of the PMT to direct the backscattering signal toward the spectrometer, which is equipped with a charge-coupled device, and then take a spectrum of the selected single GNS. Be careful that the spectrum here is a mixture of GNS scattering and background due to reflections from other surfaces.
- Switch back to the PMT detector, and take another image to confirm that the particle position has not changed. Then, shift the focus to a point at which no particle is present. Switch back to the spectrometer, and take one more spectrum, which represents the background.
- Subtract the background spectrum from step 4.5 from the spectrum from step 4.4 to obtain a clear backscattering spectrum of a single GNS.

5. Alignment of SAX Microscope

- See **Figure 3** for the scheme of the SAX microscope, where an ideal sinusoidal temporal modulation is obtained from the beat frequency between two acousto-optic modulators (AOMs). First, adjust the beam size of the laser to meet the requirement of the subsequent AOMs. Split the 532 nm laser light into two beams by using a 50/50 beam splitter.
- Guide the two beams through the two AOMs, with one beam passing through each AOM. The modulating frequencies of the two AOMs must be different. For example, one may be at 40.000 MHz and the other at 40.010 MHz, yielding a difference frequency of 10 kHz. This difference frequency will be the fundamental modulation frequency f_m for the SAX signals.
- Take the first-order diffracted beams from both AOMs, and combine the two beams by using another 50/50 beam splitter. Adjust the mirrors after the AOMs to collimate the two beams.
- Add a photodetector that is connected to an oscilloscope to monitor the temporal modulation. Split a small portion of the laser with a slide glass, and send it to the photodetector, as shown in **Figure 3**. With correct modulation and beam overlapping, observe sinusoidal intensity modulation at the main frequency f_m , similar to that of the waveform shown in **Figure 4**.
Note: The background of the modulation needs to be as low as possible to achieve the maximum modulation depth. In addition, use the Fourier analysis function of the oscilloscope to check that the harmonic distortion of the modulation is diminishing. To achieve successful SAX implementation, ensure a perfect sinusoidal excitation intensity modulation with minimized initial nonlinearity.
- Disconnect the electric output of the photodetector from the oscilloscope and connect to the reference input of a lock-in amplifier.
- As shown in **Figure 3**, align the laser beam into the confocal system following the previous protocols. Here, connect the electric output of the PMT to the lock-in amplifier as the signal input.
- Use a blank cover glass as the sample, and check the linearity of the electric detection system by gradually increasing the excitation power, as shown in **Figure 5**, where the detector is linear below a readout value of 1-V. In all subsequent measurements, take care to restrain the readout to below this value.

8. Set the output of the lock-in amplifier to export the absolute magnitude of the voltage signal. By changing the harmonic component setting at the reference channel, obtain the amplitudes of the SAX signals, A_1 , A_2 , and so on.
9. Export the linear and nonlinear signals from the lock-in amplifier to a data acquisition card, which also receives the driving voltage signals of the raster scanning galvano mirrors. With the aid of a customized Labview program, synchronize the signals of the lock-in amplifier and the galvano mirrors to form an image.
10. To optimize the signal-to-noise ratio in the images, select appropriately the pixel acquisition and integration times of the lock-in amplifier. For example, when the main modulation frequency f_m of the excitation is 10 kHz, that is, when the period is 100 μ sec, set the integration time of the lock-in amplifier to be at least three times longer than the period. Adding the time of the galvano mirror movement, the acquisition speed is set at 1,500 pixels per second in the SAX imaging mode.

Representative Results

Figure 6 shows the measured spectrum from an 80 nm GNS. A calculated curve based on the Mie theory is given in the same plot, showing excellent agreement. The LSPR peak is around 580 nm. In the following experiment, the laser wavelength was 532 nm, which was chosen as it is located inside the plasmonic band to enhance optical scattering with plasmonic effect and enable scattering saturation²¹.

Figure 7 presents scattering images of a single gold nanoparticle at different excitation intensities, and the bottom row provides the line profile of each particle to highlight the nonlinearity. The image size is 600 nm $\times$ 600 nm, and the pixel size is 13.8 nm. The acquisition speed was 234,000 pixels per second in the normal xy imaging mode. Each image was averaged over five acquisitions to enhance the signal to noise ratio.

When the excitation intensity is lower than 1.5×10^6 W/cm², the scattering is linearly dependent on the excitation intensity, so the resulting image of a single nanoparticle resembles the PSF of the excitation beam, with a standard Gaussian profile. However, when the excitation intensity increases to 1.7×10^6 W/cm², not only clear flattening at the top of the PSF is observed, but also widening of the FWHM, indicating saturation. Very interestingly, at slightly higher intensities, the central intensity becomes lower than the peripheral, resulting in a donut-shaped PSF. Then, as the excitation intensity continues to increase, the scattering intensity increases again, revealing reverse saturation and resulting in a new peak in the center of the PSF.

By plotting the central intensities of the PSFs at different excitation intensities, the scattering intensity dependence is obtained, as shown by the dots in **Figure 8**. This curve clearly reveals the trends of saturation and reverse saturation behaviors. As expected, it looks very similar to the intensity dependence of nonlinear absorption¹⁵⁻¹⁷. Following the typical method of analyzing nonlinear absorption, a polynomial function was used to fit the nonlinear scattering result. However, different from most nonlinear absorption studies, in which third-order nonlinearity is sufficient to model the results, here fifth-order nonlinearity was required to better fit the scattering curve.

As mentioned in section 5, the harmonic frequency components can be experimentally extracted by a lock-in amplifier, and the results are given in Figure 9A. On the other hand, the harmonic components can be calculated from Figure 8. First, use a polynomial function, where I is excitation intensity, to fit Figure 8, so we have the fitting parameters α , β , γ ... We can then express the excitation intensity as a temporally modulated function $I(t) = I_0(1 + \cos(2\pi f_m t))/2$, where t is time, f_m is the modulation frequency, and I_0 is the maximum excitation intensity. By substituting $I(t)$ into $S(I)$, and make a Fourier transform to convert the resultant $S(I(t))$ into frequency domain, we have the following equation composed of multiple delta functions (δ):

$$\begin{aligned} FT[S(I(t))] &= \alpha FT[I(t)] + \beta FT[I(t)^2] + \gamma FT[I(t)^3] + \dots \\ &= A_0\delta(\omega) + A_1\delta(\omega - 1f_m) + A_2\delta(\omega - 2f_m) + A_3\delta(\omega - 3f_m) + \dots \end{aligned} \quad (3)$$

The coefficient of each delta function (A_0 , A_1 , A_2 , etc.) represents the amplitude of the SAX signal at the corresponding harmonic frequency. These coefficients, which correspond to the SAX signal strengths at different harmonics, can be written as functions of the fitting parameters α , β , γ ...

$$\begin{aligned} A_1 &= \sqrt{\frac{\pi}{2}} \left(\frac{1}{2} \alpha I + \frac{1}{2} \beta I^2 + \frac{15}{32} \gamma I^3 + \dots \right) \\ A_2 &= \sqrt{\frac{\pi}{2}} \left(\frac{1}{8} \beta I^2 + \frac{3}{16} \gamma I^3 + \dots \right) \\ A_3 &= \sqrt{\frac{\pi}{2}} \left(\frac{1}{32} \gamma I^3 + \dots \right) \end{aligned} \quad (4)$$

The calculation results are shown in **Figure 9B**. The experimental and calculation plots agree closely, especially in the following two aspects.

First, the curves of $2f_m$ and $3f_m$ are not smooth, showing dips at specific intensities along the curves. In both figures, there are three dips in the $2f_m$ curves, while two dips are seen in the $3f_m$ curves. Second, the slopes are different with different excitation intensities. When the excitation intensity is not high, the slopes of $1f_m$, $2f_m$, and $3f_m$ are 1, 2, and 3, respectively. However, after each dip, the slopes of the corresponding nonlinear curves become larger.

With the dips and slope variations, unconventional PSFs are anticipated if the nonlinear components are extracted via the SAX technique, when the excitation intensity increases across the dips. **Figure 10A** shows the SAX image examples of the $1f_m$, $2f_m$, and $3f_m$ frequency components at different excitation intensities. In the first row, the excitation intensity is 0.7 MW/cm², which is sufficient to induce the nonlinear components, but the amplitude is relatively weak. At this intensity level, the slope of the $2f_m$ signal is 2, and it is 3 for the $3f_m$ signal, as shown in **Figure 9A**. If the excitation intensity increases to the level of the first dip of the $2f_m$ signal, the SAX images of the $2f_m$ signal become donut shaped, as shown in the second row in **Figure 10A**. Both the $1f_m$ and $3f_m$ images remain solid, while the FWHM of the $3f_m$ PSF is significantly smaller than that of the $1f_m$ signal, manifesting remarkable resolution enhancement. From the signal profile at the rightmost panel of the same row, the FWHM of the $2f_m$ donut ring is about 110 nm. On the other hand, the third row of **Figure 10A** shows that when the excitation intensity increases to the first dip of

the $3f_m$ signal, only the $3f_m$ image becomes donut shaped, with a 65 nm ring width. At this intensity, remarkable resolution enhancement is found when comparing the $2f_m$ signal to the $1f_m$ one.

Figures 10B and **10C** show the calculated PSFs of the $2f_m$ and $3f_m$ signals, respectively, at the corresponding intensities that result in the donut shapes. The calculations were based on the polynomial fitting curve in **Figure 9B**. The calculated curves well reproduce the features of the experimental PSFs in the rightmost panels in **Figure 10A**, confirming again the suitability of a fifth-order polynomial fit for the nonlinear scattering.

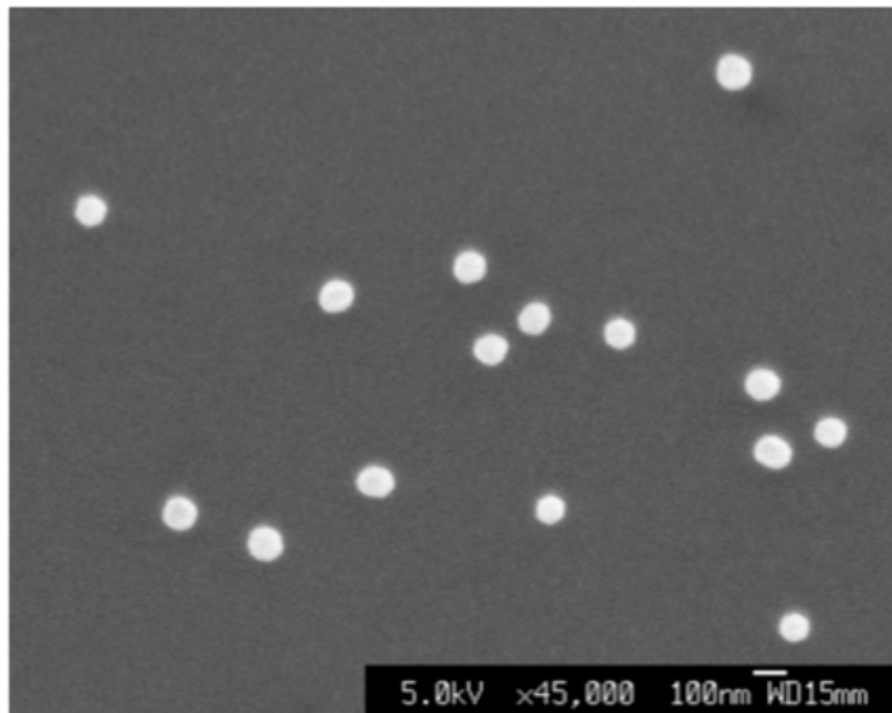


Figure 1. SEM image of GNSs. By performing the preparation processes described in the first part of the protocol, sufficiently separated GNSs are observed. With more than 100 nm between GNSs, their LSPR effects are not coupled to each other. Scale bar: 100 nm. [Please click here to view a larger version of this figure.](#)

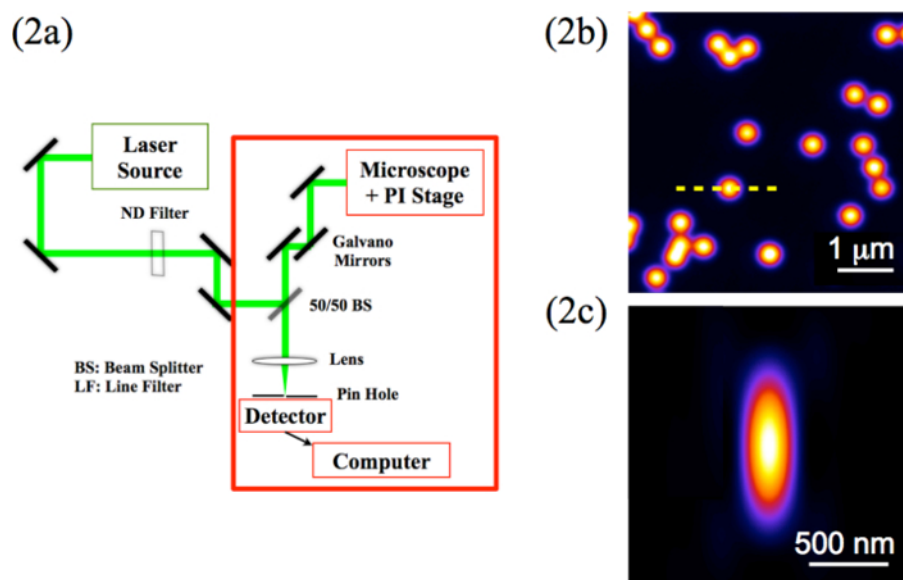


Figure 2. (A) Setup of home-built confocal microscope²⁴. (B) xy image with GNSs at focus. 2(c): xz image of PSF with correct alignment. There are two laser sources for this system. One is a 532 nm continuous-wave laser, and the other is a pulsed super-continuum laser. When measuring the scattering signals, a 532 nm continuous-wave laser was used as the source and a PMT as the detector (with a laser line filter inserted). To measure the spectrum, a super-continuum laser was adopted as the laser source and a spectrometer as the detector. The selected laser is sent through a set of neutral density filters to control the excitation intensity. A 50/50 beamsplitter guides the laser into the scanning microscope and allows half of the backward-scattering signals into the PMT or the spectrometer, which is selected by a flipping mirror. In the scanning system, there are two galvano mirrors that form vertical and horizontal raster scanning in the focal plane of an objective. The backward scattering is collected by the same objective and converted into electrical signals by the detectors. The signals are synchronized with the confocal scanning system to form images. The PI stage was used to acquire the xz image by moving the GNSs axially. [Please click here to view a larger version of this figure.](#)

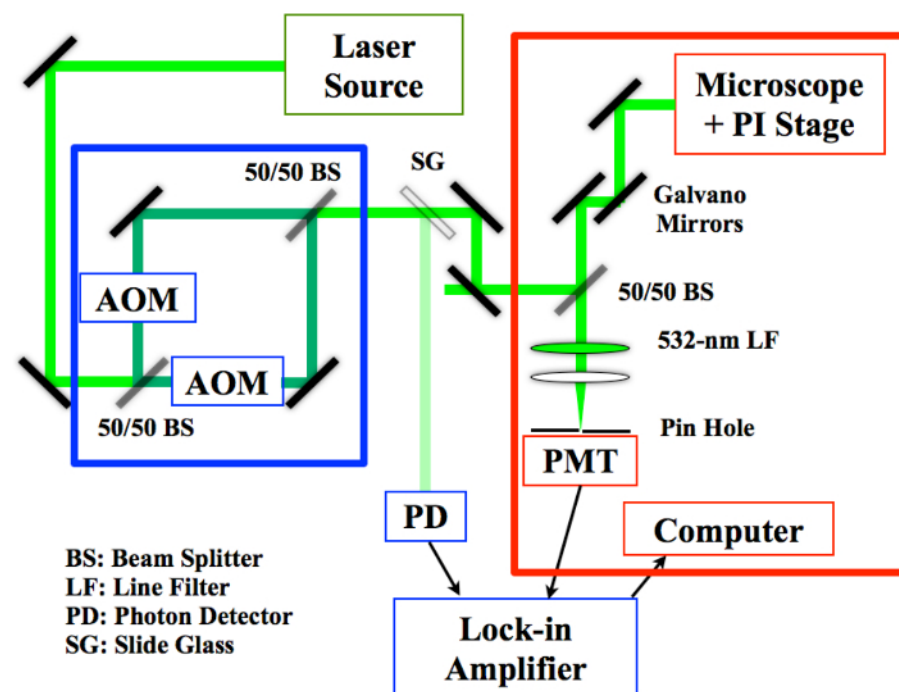


Figure 3. Setup of SAX microscopy. Most components are the same as those obtained from a confocal microscope (red rectangle), but sinusoidal modulation was added to the excitation laser beam. Blue rectangle shows modulator setup. First, the excitation laser was divided into two beams and separately sent through two AOMs to produce high-frequency modulations with slightly different frequencies. Then, the two modulated beams were combined to produce sinusoidal modulation at the beat frequency between the two AOMs. [Please click here to view a larger version of this figure.](#)

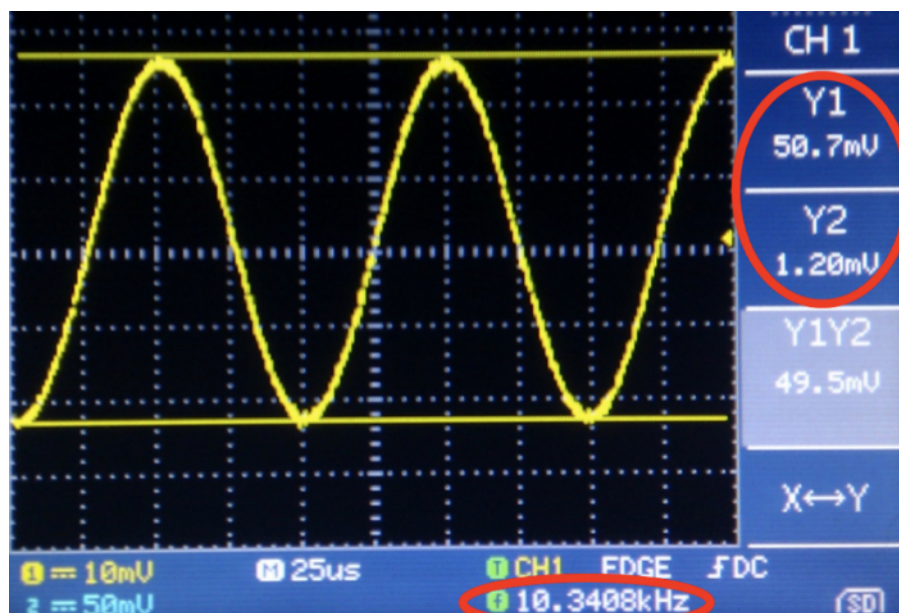


Figure 4. Modulation of combined beams after AOMs measured by oscilloscope. Y1 and Y2 indicate maximum (52.1 mW) and minimum (1.2 mW) values of modulation intensity, respectively. Y2 should be zero to achieve perfect modulation. Current modulation frequency was 10 kHz. [Please click here to view a larger version of this figure.](#)

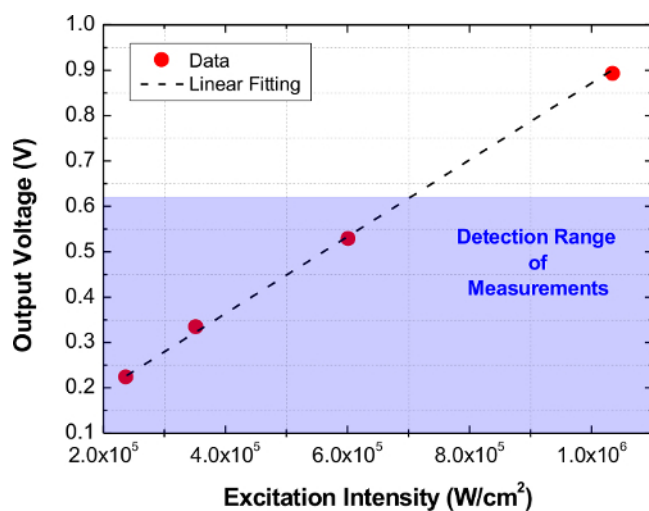


Figure 5. Linearity test of detection system. By placing a cover glass at the focal plane, the reflection of the excitation laser from the glass/air interface was used to check the linearity of the detection system. The signal output versus excitation intensity shows linearity below a readout value of 1-V. Furthermore, the noise level is well below 10^{-4} V, so the system provides a dynamic range of at least 10^4 . [Please click here to view a larger version of this figure.](#)

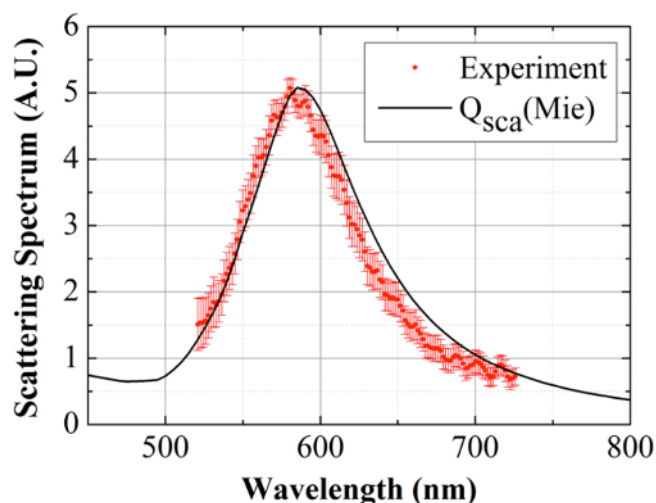


Figure 6. Scattering spectrum of 80 nm GNS. Red dots indicate experimental measurements, and black line represents calculation from Mie theory. [Please click here to view a larger version of this figure.](#)

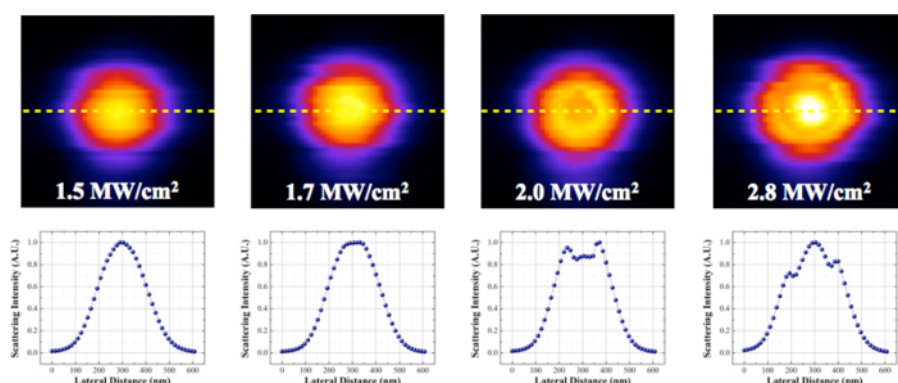


Figure 7. Scattering images of GNS from linear to reverse saturation. Top row shows backscattering images, and bottom row gives signal profiles of selected nanoparticle at various excitation intensities. Transition from linearity to saturation to reverse saturation is clearly observed. [Please click here to view a larger version of this figure.](#)

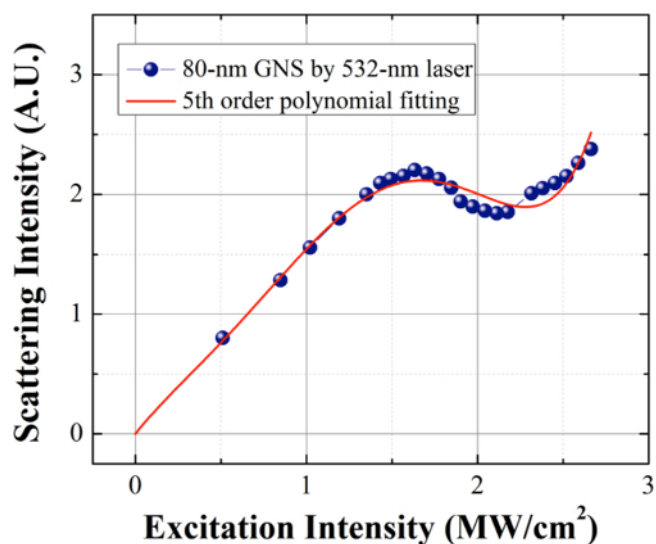


Figure 8. Scattering intensity versus excitation intensity from single GNS. Blue dots correspond to scattering intensities at center of PSF at different excitation intensities, showing very nonlinear responses, including saturation and reverse saturation. Red curve indicates fit curve based on fifth-order polynomial function. (Images reproduced from Ref. ²⁵.) [Please click here to view a larger version of this figure.](#)

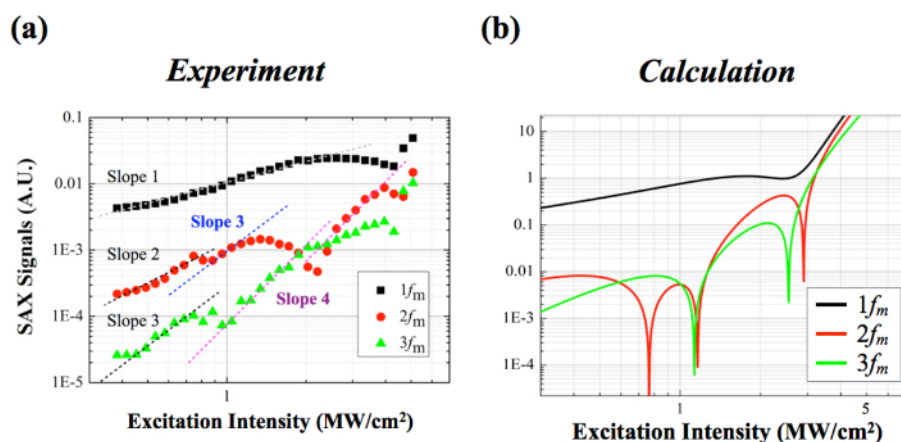


Figure 9. Intensity dependences of SAX signals according to (A) experiment and (B) calculation. (A) SAX signals were extracted by lock-in amplifier, and each experimental data point was averaged over four 80 nm GNSs. Dotted lines indicate slopes of SAX signals²⁵. (B) Following protocol 5, SAX signals were calculated based on fifth-order polynomial fit in Figure 8. (Images reproduced from Ref. ²⁵) [Please click here to view a larger version of this figure.](#)

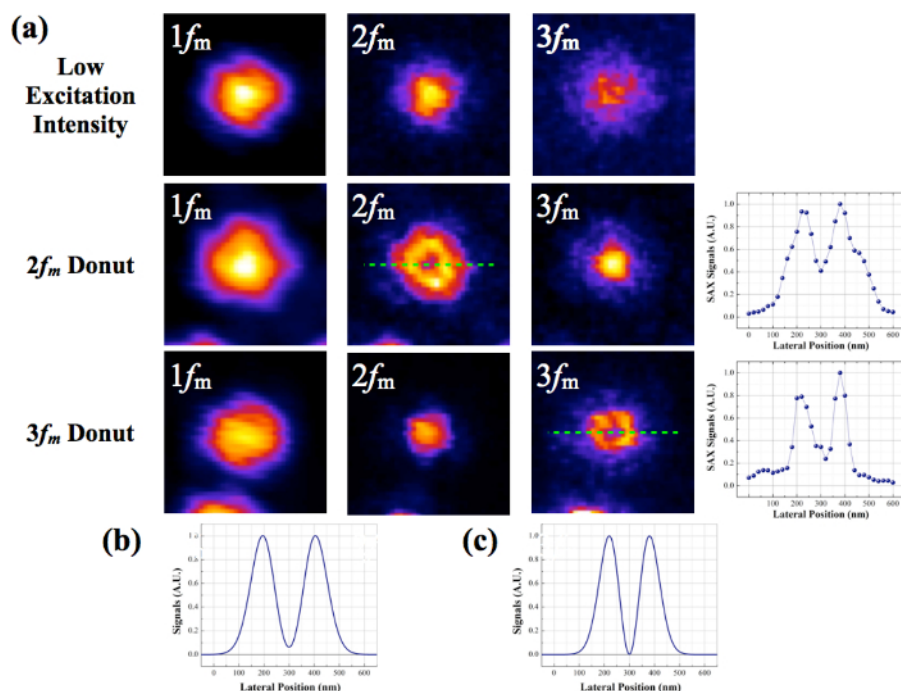


Figure 10. SAX images at different excitation intensities. (A) Experimentally observed $1f_m$, $2f_m$, and $3f_m$ SAX images at different excitation intensities. Pixel size is 20 nm, and each image size is 750 nm $\times$ 750 nm. Intensity profiles of donuts at $2f_m$ and $3f_m$ are plotted in rightmost panels. (B) Calculated image profile of $2f_m$ image at 0.75 MW/cm². (C) Calculated image profile of $3f_m$ image at 1.1 MW/cm². (Images reproduced from Ref. ²⁵.) [Please click here to view a larger version of this figure.](#)

Discussion

In the protocol, there are several critical steps. First, when preparing the samples, the density of nanoparticles should not be too high, to avoid plasmonic coupling among particles. If two or more particles are very close to each other, the coupling results in the LSPR wavelength shifting toward longer wavelengths, thus significantly reducing the nonlinearity. However, this imaging technique actually maps the distribution of plasmonic modes, instead of the particles themselves. Therefore, it is expected that with an appropriate excitation wavelength, the coupled plasmonic modes can also show strong scattering nonlinearity and can be imaged with enhanced resolution. Second, it is very important to produce pure sinusoidal modulation within the excitation beam, thus motivating the use of beating between the two AOMs. Since resolution enhancement relies on extracting nonlinear parts (harmonic frequency components) of the scattering signal modulation, if nonlinear distortion is present in the excitation modulation, then extraction will be more difficult. In addition, in the current scheme, an interferometer setup is used to produce the beating modulation, so the alignment of the two beams in the interferometer is also critical to achieve as large of a modulation depth as possible. Third, it is very important to ensure that the signal nonlinearity does not arise from the detection system (which includes the detector, amplifier, A/D converter, and computer I/O). Therefore, special attention is necessary to guarantee that the detection system is working within the dynamic range. The dynamic range is defined as the region of detection system linearity, that is, from the noise level to detector saturation. In the current case, the detected voltage signal is linear below 1 V, and the noise level is below 10^{-4} V. Therefore, the system provides a dynamic range

of at least 10^4 . To ensure that the signal nonlinearity originates from the gold nanoparticle itself, not from the detection system, it is necessary to maintain the readout value within the dynamic range. The fourth critical factor is the mechanical stability of the sample. During the nonlinearity characterization, it is essential that the nanoparticles remain in the same focal plane. Axial drift of the nanoparticle or the sample stage would severely affect the accuracy of the nonlinearity assessment. Therefore, when working with nanoparticles, it is important to find particles that do not easily move around under light excitation. On the other hand, it is also possible to work with samples grown from lithography. In this case, microscopic stage stability is the main limiting factor. There are stages with position feedback control that can greatly enhance the stability. Alternatively, since stage movement is typically very slow (e.g., 1 μm in 10 min), it is helpful to acquire a xyz 3D image stack, such as 10 images with 100 nm axial separation between adjacent images, at each different intensity value. Then during the analysis stage, the brightest image out of each stack should be chosen as the representative image at that intensity.

In principle, the resolution of saturation-based techniques, which include SAX and saturated structured-illumination microscopy (SSIM)²⁶, exhibits no lower limit as long as high-order nonlinearity (high harmonic frequency components) can be achieved. Nevertheless, in practice, the resolution is limited by the signal-to-noise ratio (SNR), especially when extracting higher-order harmonic demodulation components. There are a few strategies that can enhance the SNR. For example, it has been shown that the modulation frequency severely affects the SNR²⁷. It is also possible to enhance the SNR by calculating the intensity difference between non-saturated and saturated signals to extract only the saturated signal (manuscript in preparation).

Now we make a brief comparison of the current technique to others on two aspects: contrast and resolution. As mentioned, the contrast of our images reflects the plasmonic mode distribution, or equivalently the nonlinear plasmonic local density of state (LDOS). It is well known that electron energy loss spectroscopy (EELS) or two-photon photoluminescence (TPPL) can also be used to probe the LDOS. Compared to EELS, our optical imaging method allows the potential of mapping LDOS of metal nanospheres in biological samples. Compared to TPPL, our technique provides superior resolution. On the other hand, compared to other existing methods that achieve resolution beyond the diffraction limit, the main achievement of this work is the development of a novel non-bleaching contrast method for super-resolution microscopy. Most of the previous far-field super-resolution techniques have relied on nonlinearities of fluorophores, including their on/off switching²⁸⁻³⁰, or by saturation of fluorescence emission^{22,26,31}. However, fluorescence exhibits an intrinsic problem of photo-bleaching, especially under strong light illumination. This study demonstrated that saturable scattering of GNSs is a promising method of super-resolution microscopy since there is no bleaching issue²¹. Compared with previous studies of SAX microscopy utilizing fluorescence, the resolution enhancement with saturable scattering was much higher in this investigation, possibly due to the higher-order nonlinearity²². In addition, other than SAX microscopy, there is another super-resolution technique based on saturation: SSIM²⁶. SSIM exploits spatial modulation of fringes to extract the nonlinear signals, while SAX microscopy utilizes temporal modulation. With the saturation property of this non-bleaching scattering, it is therefore expected that this discovery can be combined with SSIM to improve the spatial resolution under wide-field illumination.

In future applications, this plasmonic SAX technique will be useful not only to resolve resonance-mode distributions and dynamics in plasmonic circuits, but also to enhance the resolution of biological tissue imaging. Similar resolution enhancement has been demonstrated with other plasmonic materials such as silver (unpublished), as well as non-plasmonic materials, such as silicon³². In the super-resolution imaging field, SAX microscopy has advantages in several respects. Compared to stochastic optical reconstruction microscopy (STORM) and photo-activated localization microscopy (PALM), SAX microscopy has a faster scanning speed of only a few seconds per image. Compared to stimulated emission depletion (STED) microscopy, only one laser is required for SAX microscopy, significantly reducing the optical complexity. Compared to SSIM, the resolution of SAX is simultaneously improved in both the lateral and axial directions. In addition, to achieve a sufficient imaging depth, random scattering along the beam path of excitation or collection is critical. For wide-field techniques like STORM, PALM, and SSIM, images are captured with a camera, which is highly susceptible to random scattering of emitted fluorescence photons in tissues. For point-scan techniques like STED and SAX, the fluorescence signals are collected by a point detector, so they are more robust against tissue scattering. Nevertheless, STED requires a phase plate to create a donut beam profile at the focus, and the phase information may be deteriorated during beam propagation in tissues. Therefore, SAX microscopy should be the best among these modalities for deep tissue super-resolution imaging.

Disclosures

The authors declare that they have no competing financial interests.

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