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Spectral Properties and Dynamics of Gold Nanorods Revealed by EMCCD-Based Spectral Phasor Method

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ABSTRACT Gold nanorods (NRs) with tunable plasmon-resonant absorption in the near-infrared region have considerable advantages over organic fluorophores as imaging agents due to their brightness and lack of photobleaching. However, the luminescence spectral properties of NRs have not been fully characterized at the single particle level due to lack of proper analytic tools. Here, we present a spectral phasor analysis method that allows investigations of NRs’ spectra at single particle level showing the spectral variance and providing spatial information during imaging. The broad phasor distribution obtained by the spectral phasor analysis indicates that spectra of NRs are different from particle to particle. NRs with different spectra can be identified in images with high spectral resolution. The spectral behaviors of NRs under different imaging conditions, for example, different excitation powers and wavelengths, were revealed by our laser-scanning multiphoton microscope using a high-resolution spectrograph with imaging capability. Our results prove that the spectral phasor method is an easy and efficient tool in hyper-spectral imaging analysis to unravel subtle changes of the emission spectrum. We applied this method to study the spectral dynamics of NRs during direct optical trapping and by optothermal trapping. Interestingly, different spectral shifts were observed in both trapping phenomena.

INTRODUCTION

In recent years, various gold nanostructures have been developed and studied due to their unique optical imaging properties and biocompatibility, including nanospheres, nanoshells, nanorods, nanocubes, nanocages and more (Conde et al., 2012; Gao et al., 2014; Kawamura et al., 2013; Tong et al., 2009). Gold nanostructures can enhance the local electromagnetic field amplitude and significantly increase the quantum yield by the surface plasmon resonance effect (SPR) (Klar et al., 1998; Mayer and Hafner, 2011; Messersmith et al., 2013). These optical properties can be finely controlled by varying the size, shape, compositions, and local environments (Gao et al., 2014; Kawamura et al., 2013; Kelly et al., 2002; Slaughter et al., 2010). Furthermore, their surfaces can be easily biodecorated for different biomedical applications (Durr et al., 2007; El-Sayed et al., 2005; Murphy et al., 2008; Sokolov et al., 2003). These properties make them ideal as imaging and sensing agents in many biomedical applications (Anker et al., 2008; He et al., 2008; Tong et al., 2009).

Among them, gold nanorods (NRs) have a longitudinal surface plasmon resonance (LSPR), corresponding to electron oscillation along the long axis of the rods, whose wavelength can be easily tuned throughout the near-infrared (NIR) region, for example, from ~600 nm to 1400 nm by varying the aspect ratio (Fang et al., 2012; Link et al., 1999; Murphy et al., 2005). Excitation at LSPR can greatly enhance the luminescence from NRs and improves the imaging quality accordingly. This excitation wavelength range is attractive for in vivo imaging due to better penetrations that can be achieved by NIR excitation. Many applications in vitro and in vivo of two-photon luminescence imaging of gold NRs have been reported (Durr et al., 2007; Wang et al., 2005; Wang et al., 2013) and demonstrated that NRs have great potential as imaging contrast agents for biomedical diagnosis. Furthermore, high optothermal conversion efficiency has been reported, that is, over 96% of the absorbed photons can be converted into heat by nonradiative electron relaxation (Link et al., 2000; Tong et al., 2009), so NRs can also be used as photothermal agents in localized NIR-induced hyperthermia (Chou et al., 2005; Hu et al., 2009; Huff et al., 2007; Tong,...)
et al., 2009). Optical trapping and manipulation of single NRs has also been reported (Deng et al., 2012; Gu et al., 2014; Lin et al., 2012; Pelton et al., 2006; Selhuber-Unkel et al., 2008; Toussaint et al., 2007). It has been discussed extensively that the localized LSPR effect provides strong electromagnetic (EM) field enhancement, which may enhance EM trapping forces. The thermal effect due to LSPR has a significant impact in determining the final outcome of the nanoparticle trapping (Selhuber-Unkel et al., 2008; Wu and Gan, 2010). For example, Pelton et al. (2006) demonstrated three-dimensional trapping and alignment of single NR by using wavelengths slightly detuned from LSPR. Gu et al. (2014) showed optical manipulation of NRs by using optical nonlinear endoscopy via the optothermal attracting force from the dynamic increase in the environmental temperature around the trapped NRs at LSPR. The large trapping range is likely due to the thermal force that decays slower and it has a much broader working range than the EM force (Wu and Gan, 2010). The optical trapping and manipulation of NRs offer great opportunities in nanoscale architecture and target drug delivery.

Most imaging studies and applications of gold NRs are based on their bright emission in the visible range during one or multiphoton excitations. Furthermore, the emission of NR can be controlled and tuned by its physical properties, imaging conditions and even local environments. For example, it has been reported that the emission spectrum was influenced by different excitation modes and wavelength as well as dielectric environment during one photon excitation (Wackenhut et al., 2013). Therefore, understanding the spectral dynamics of NRs becomes important during imaging studies. So far the luminescence spectral information of NRs during multiphoton imaging is still limited (Bouhelier et al., 2005; Imura and Okamoto, 2009; Wang et al., 2013), possibly due to lack of proper analysis tools to study emission spectra in a global manner from single particles to bulk. NRs have a broad emission in VIS range and defining a specific spectral component is not straightforward. In addition, the hyper-spectral imaging of NRs in a laser-scanning microscope usually generates large data sets, for example, typical spectral images (256 × 256 pixels with 512 points/pixel-spectrum) have ~64 k spectra in one image. It is not easy to globally display such a large data set and resolve the spatial and spectral information at single particle level. All these issues can be alleviated by using the spectral phasor approach. The phasor approach in fluorescence lifetime imaging microscopy (FLIM) is a global analysis method (Digman et al., 2008) and it provides fast, global, graphical, and quantitative analysis of decay curves. This approach was expanded by Fereidouni et al., (2012) from fluorescence lifetime to the spectral domain and successfully applied in living cell studies (Andrews et al., 2012). In the phasor analysis, the spectral profile of each pixel in one spectral image is Fourier transformed to produce two co-ordinates represented in the spectral phasor plot. The angular coordinate is proportional to the spectral center of mass; the radial coordinate is inversely related to the spectral width. A red shift in wavelength will lead to an increase of the angular position, whereas a decrease in spectral width will increase the radius of the phasor. Hence, each pixel with certain spectrum is transferred into one point in the 2-D phasor plot without demixing basis spectra, which requires prior knowledge. A cluster of points in the phasor plot corresponds to pixels with similar spectra. Components with different phasors can be identified in the corresponding spectral image. Such separation offers the opportunity to recognize different population/structures in target samples with different spectra. Therefore, the spectral phasor analysis is an ideal tool for hyper-spectral imaging analysis.

Here, we show that the spectral phasor method can provide high spectral resolution (0.5 nm shift), fast data acquisition (up to 7 second per spectral image) and an easy graphical data analysis of spectral images both in spectral domain and in spatial domain. Using this approach, we studied spectral properties of NRs under different excitation conditions, that is, different excitation powers at LSPR wavelength and different excitation wavelengths. We observed spectral shifts which are due to the population change of NRs with different spectra. Spectra of NRs undergoing direct optical trapping and the optothermal trapping were investigated. We found that spectra of trapped NR changed upon heating during the direct trapping, whereas spectra of NR clusters on glass are red shifted during the optothermal trapping. Our results demonstrate that the spectral phasor method has the sensitivity to reveal subtle changes of emission spectra and can assign these changes to specific regions of an image.

**EXPERIMENTAL SECTION**

**Instrument Setup**

In previous applications of the spectral phasor approach, two types of instruments have been described. Fereidouni et al. used the prism-based spectrograph combined with 128 channels EMCCD to acquire spectral imaging in 6.5 s (Fereidouni et al., 2012). Cutrale et al. used a built-in grating with a 32-channel PMT in the Zeiss LSM710 (Cutrale et al., 2013). In order to obtain higher spectral resolution with better linearity in the spectral range, we used a grating-based spectrograph (Andor SR303i) with a 512-channel ultrafast EMCCD (Andor iXon Ultra). A typical laser-scanning hyper-spectral image with ~647 k spectra can be acquired in about 7s. The spectral data set was then imported and analyzed by the spectral phasor analysis (SimFCS software, available from http://www.lfd.uci.edu/).

Multiphoton luminescence hyper-spectra of gold NRs obtained with a laser-scanning microscope are shown in Figure 1. Briefly, the NIR beam from a MaiTai HP Ti:sapphire laser is coupled into a commercial inverted Olympus FY1000 confocal system. The laser is equipped with a DeepSea unit to compensate for group velocity dispersion. The laser power is controlled by an acoustic optical modulator. The laser beam is then expanded in order to fulfill the back aperture of the objective lens and coupled into Olympus laser-scanning unit via the NIR port. A RDM690 excitation dichroic mirror reflects the excitation beam into the scanning path and allows the collected signal to reach the detectors in the descanned path. A 60× water objective (UPlanSApo, Microscopy Research and Technique
NA = 1.2) is used to focus the excitation beam to the sample and collect the emission signal. The luminescence signal in the descanned path is coupled into a 200 μm fiber at the fiber output of Olympus unit. Due to the intrinsic 3-D capability of multiphoton excitation, the confocal pinhole is fully open during measurements.

The emission signal out of the fiber is delivered to the spectrograph and dispersed by one grating (50 l/mm, 600 nm blaze). The spectrum is focused to a center area of 512 × 30 pixels in the EMCCD. The Olympus FV1000 scanner is driven by our own controller (IOTECH DAQboard 3001) and software (SimFCS) to gain the full control of scanning pattern and timing. EMCCD frame acquisition is synchronized with pixel clock and starts the spectral acquisition once the start frame trigger signal is received. For a 256 × 256 spectral image, ~64 k spectra (512 channels/pixel-spectrum) are collected. The exact number of spectra collected depends on the scanning waveform retracing. The spectral image can be acquired as fast as 7 s with ~100 μs/pixel dwelling time. A background spectral image is collected before any experiment to remove the camera dark noise during data processing. Data are then imported into the SimFCS software, where images are reconstructed and spectra are extracted. Spectra are corrected with the actual spectral response curve of the system obtained using a calibrated tungsten lamp (the black curve in Fig. 1). The phasor approach is used to analyze this large spectral data set. Selection of certain phasor clusters in the phasor plot, for example, a green circle, is then used to color code corresponding pixels in the image. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 1. Schematic of the EMCCD-based spectral phasor system. The laser beam from the MaiTai HP Ti:sapphire laser is coupled into a commercial Olympus FV1000 confocal system with IX81 body. A DeepSee unit is equipped in front of MaiTai to compensate for group velocity dispersion. Laser power is controlled by an acousto-optic modulator (AOM). The beam is expanded and sent to the IR port of Olympus scanning box. The NIR laser beam is reflected into scanning path by a RDM600 dichroic mirror. The Olympus scanner is driven by our own scanning controller (IOTECH DAQboard3001) and software (SimFCS) to gain the full control of scanning pattern and timing. The laser beam is finally focused by a ×60 water objective (Olympus, UPlanSApo, NA = 1.2). Signal is collected by the same objective and sent to the descanned path. The confocal pinhole is fully open for multiphoton excitation. A 200 μm fiber is used to collect the signal at the far port of Olympus confocal box and delivers the signal into a spectrograph (Andor, Shamrock SR-303i). The spectrum is then focused on an EMCCD (Andor, iXon ultra) at a center area of 512 × 30 pixels. The EMCCD is synchronized with the scanning pattern of our controller and starts the spectral acquisition once the pixel clock is received. For an image of 256 × 256 pixels, ~67 k spectra were collected and the exact number of spectra depends on the scanning waveform retracing. A full spectral image can be acquired as fast as 7 s with 100 μs dwelling time and processed by SimFCS software, where the image is reconstructed and spectra are extracted (the blue curve) and corrected (the red curve) with the system response (the black curve). The phasor approach is used to analyze this large spectral data set. Selection of certain phasor clusters in the phasor plot, for example, a green circle, is then used to color code corresponding pixels in the image. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
spatial image. By associating clusters of spectral phasors with pixels in the image, we are able to identified components/species in the image based on the spectral properties.

### Sample Preparation

CTAB-capped gold nanorod with surface plasma resonance (SPR) at 840 nm was purchased from Nanopartz (Loveland, CO). Axial diameter is 10 nm and the length is 44 nm. Thus, the aspect ratio is 4.4. The stock concentration is $5.7 \times 10^{11}$ nanoparticles/mL. During measurements, 200 μL solution was sonicated for 1 hour before added into an 8-well glass bottom chamber. Spectral images usually were taken 30 mm above the bottom surface. In optical trapping studies, stock solution was diluted 10 times with milli-Q water, that is, $5.7 \times 10^{10}$ nanoparticles/mL final concentration, and sonicated for 1 hour before measurements.

### RESULTS AND DISCUSSION

#### Characteristics of the Spectral Imaging System

One advantage of spectral phasor method is the graphic identification of different spectral center of mass and spectral widths with high resolutions. It has been previously reported using simulations that 2 nm in spectral width change or spectral shift can be correctly recognized by first and second harmonics phasor with a low-resolution instrument (32-channels/spectrum) (Golfetto, 2013).

In order to confirm the sensitivity of our system is sufficient for most imaging studies, especially in spectral shift, we acquired second harmonic generation (SHG) spectra from urea crystals by changing the excitation wavelength from 930 nm to 940 nm with 1 nm step. The 50 L/mm grating covers 532 nm on 512 channels of EMCCD and leads to 1.04 nm pixel bandwidth which is a big improvement than previously reported 32-channels PMT with 9.7 nm bandwidth. The SHG spectra in the range from 465 nm to 470 nm are shown in Figure 2A. It is clear that 0.5 nm difference is not distinguishable in the raw spectral plot. However, after phasor transformation, each SHG spectrum center of mass can be identified as a single spot in the phasor plot (Figs. 2B and 2C) and well separated from others. Figure 2B shows the first harmonic of the phasor transform. Figure 2C shows the second harmonic that has a smaller total wavelength range but provides better angular separation. As previously discussed by Cutrale et al. (2013) and Golfetto et al. (2013) different harmonics of the Fourier transform provide additional polar plots that reveal finer details of the spectral response. Because NRs have very broad emission spectra, we used the first harmonic for the analyses of this paper.

The wavelength resolution can be improved by using a different grating already installed in the spectrograph, that is, 150 L/mm grating which covers 176 nm into 512 channels and leads to a 0.34 nm bandwidth. The high resolution is an advantage in the situation where better spectral resolution is necessary.

To improve the image speed, the cropped sensor mode with full vertical binning of EMCCD is used during measurements. Briefly, the spectrum is focused into a 512 × 30 pixels strip in the central area of the EMCCD and no signal falls on the rest of the sensor. Only this defined area is read out with 30-rows integrated into one in the readout register. As a result, spectral acquisition speed up to 10,000 fps can be achieved. This high speed allows high-resolution spectral imaging in a normal confocal or multiphoton microscope. A limitation is the signal level and the external trigger processing circuit in the EMCCD. Currently, a spectral image with 256 × 256 pixels (512-channels/pixel-spectrum) can be obtained in about 7 s. At a low signal level, exposure time can be increased to collect more photons. We noticed that during two-photon fluorescence imaging of typical organic dyes or autofluorescence from cells, acquisition times from 10 s to 30 s are usually sufficient.
Spectral Properties of Gold NRs at LSPR

The dependence of NRs brightness on excitation wavelengths and powers has been studied (Wang et al., 2005; Wang et al., 2013); however, the spectrum of NRs under different excitation conditions has not been reported yet. In order to obtain this information, we imaged a NR suspension using our spectral imaging system. Briefly, a 256 \times 256 pixels field of view (33 \times 33 \mu m^2), which was 30 \mu m above the glass bottom, was scanned by 840 nm femtosecond laser beam with different average powers. The concentration of the sample was 5.7 \times 10^{11} nanoparticles/mL. The exposure time for each pixel was 2 ms, and 67 spectra were collected in 135 s as one spectral image. This long exposure time allows enough photons to be accumulated at low excitation powers. Figure 3A shows normalized average spectra of NRs with 0.1, 0.25, 0.5, and 1 mW excitation powers. The luminescence spectrum of NRs is broad and it spans the whole VIS range to the NIR. Two emission bands can be distinguished: one at \sim 450 nm and the other in NIR. The SHG signal is observed at 420 nm under all the excitation powers. Spectra at 0.25, 0.5, 0.75 mW excitation powers have similar shapes. The spectrum at 0.1 mW excitation has relative lower emission at \sim 450 nm and the spectrum with at 1 mW excitation shows lower emission at \sim 575 nm.

After transferring all the spectral images into one phasor plot (Fig. 3B), a phasor cluster is obtained and represents all the pixels from those spectral images. The average of all clusters is close to the center, which indicates a large spectral width. The broad distribution of phasors implies the varieties of spectra in the images, which cannot be seen by the average spectra in Figure 3A. Selection of individual phasors in the phasor plot is used to color code corresponding pixels in the images, for example, blue, green, and red circles in Figure 3B and color-coded image pixels in Figure 3C. The large populated cluster in the green circle (centered \sim 447 nm) in Figure 3B means more NRs have spectral phasors in this region, which is confirmed by Figure 3C where pixels with green-color dominate the image. However, the number of pixels with \sim 625 nm spectral phasors increases with stronger excitation powers as shown in Figure 3C. This is likely due to the laser damage caused by strong laser powers and long exposure times at each pixel. Although the laser beam was moving in the whole area, that is, 33 \times 33 \mu m^2, we noticed that NRs can be trapped and moved with the laser beam for short...
distances, which is the origin of the traces of NRs shown in Figure 3C. Thus, the heating effect may start to melt the NRs and leads to the change of rod shape and eventually change of luminescence spectra as detected by the spectral phasor approach.

We then focus on the spectral phasors with 0.5 mW excitation power and select larger areas with green (center at 454 nm) and red (center at 620 nm) circles in Figure 3D to include more NRs in the image, which is color-coded in green and red (Fig. 3E). To confirm the observed difference, two 16 × 16 pixels regions of interest (ROI) with different color are chosen in Figure 3E and the average spectra are shown in Figure 3F. ROI “a” has a strong peak in the NIR range and a SHG signal at 420 nm. In ROI “b,” NRs only show a peak at 450 nm range with a SHG signal. This association between the spectral phasor and the spatial distribution provides a convenient way to separate the spectral behaviors of NRs in the images.

Spectral Properties of Gold NRs with Different Excitation Wavelengths

As imaging agents, gold NRs typically show a broad NIR absorption, for example, 840 nm NRs can have an absorption range from 740 to 1040 nm with peak at 840 nm (http://www.nanopartz.com). Although at the LSPR wavelength, NRs show the best efficiency in photon conversion, other wavelengths far from LSPR are also used in various applications, for example, 1200 nm has been used to imaging NRs for better penetration depth and less heating effect (Balla et al., 2011). The spectral properties of NRs with different excitation wavelengths could be important for imaging applications.

We acquired spectral images of NRs in solution under 0.5 mW average excitation power at seven different excitation wavelengths (740, 800, 840, 900, 840, 1000, and 1040 nm). These wavelengths cover the absorption range of 840 nm NRs and the tuning range of a Ti:sapphire laser. The exposure time for each pixel was 2 ms, and ~67 k spectra were collected in 135 s in one spectral image. We noticed that excitation wavelength away from the LSPR resulted in decreased emission (data not shown) as predicted (Wang et al., 2005; Wang et al., 2013).

Interestingly, the average spectra changes with different excitation wavelengths (Fig. 4A). First, SHG signals are observed at all the excitation wavelengths, except 740 nm, which is likely due to the optical cut at ~390 nm of our system. SHG peaks provide an additional imaging modality of gold NRs. However, SHG signals are mixed with luminescence. The luminescence from NRs actually dominates the spectrum.
although it has been reported that SHG and third harmonic generation from NRs are also greatly enhanced by LSPR (Hubert et al., 2007; Schwartz and Oron, 2009). Second, we separate the spectrum from 390 to 670 nm (range of our interests) into three regions as following: blue (390 to 490 nm), green (490 to 590 nm), and red (590 to 670 nm). Figure 4A shows clearly that by shifting the excitation to longer wavelengths, the luminescence in the blue and green bands (in comparison to red) increases accordingly: (1) Spectra with 740 nm and 800 nm excitation show strong peaks in red and very likely extend into the NIR range. (2) A dramatic change of spectral shape is observed at LSPR wavelength, that is, 840 nm, the blue and green bands increase rapidly while the red does not. (3) Spectra at 900 nm and 940 nm excitations have similar shapes except SHG signals. They show relative weaker signals (in comparison to 840 nm excitation) in the green and red regions. (4) Spectra at 1000 nm and 1040 nm excitations have similar shapes except SHG peaks, and they are different from other spectra with relative strong signal in the blue region (in comparison to green and red).

The average spectra can be obtained from NRs in suspensions. However, the separation of spectral components from the bulk spectrum is not straightforward. These problems could be solved by the spectral phasor approach. Each pixel in spectral images was converted into a spot in the phasor plot. The phasors in Figure 4B represent the spectral distributions of all NRs in corresponding spatial images. (1) 2D clusters close to the centers indicate that the spectra are broad. (2) At 740 nm excitation, most phasors are located in the long wavelength region. The long tail along the radius implies a distribution of different spectral widths, but with similar center of spectral mass. (3) At longer excitation wavelengths, spectra of NRs are located in the short wavelength region. These results demonstrate that the multiphoton luminescence from NRs have different emission spectra with different excitation wavelengths. This information will help the optimization in imaging applications of NRs.

Fig. 5. Spectra change during direct optical trapping. The laser beam was not moving and the power was changed as indicated. The X-axis is the time axis. (A–B): Color-coded trapping image of NRs and the phasor plot with 1 mW 840 nm excitation. (C): One trapping trace in A. The intensity profile shows one NR joined and one NR left during trapping. (D–F): Color-coded spectral image and corresponding phasor plots with different trapping powers, that is, 5 mW, 10 mW, and 15 mW. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Furthermore, the phasor approach offers the capability to study the behavior of individual NRs. By loading the spectral data with different excitation wavelengths into one phasor plot, we can observe all the corresponding spectral emissions simultaneously (Figs. 4C and 4B). It is convenient to select phasors of interest by different circles, for example, green and red. In each circle, phasors are grouped, and the corresponding pixels are color coded in the spatial images. Here, green (centered ∼450 nm) and a red (centered ∼620 nm) circles are selected and the corresponding images show hidden properties.  

1. Most NRs with the 740 nm excitation are red coded. This implies that NRs have spectral phasors at ∼620 nm region.  
2. Moving the excitation to longer wavelengths causes a decrease in the number of red color-coded NRs and an increase in the number of green color-coded NRs.  
3. At 840 nm excitation, green color-coded NRs start to dominate the spatial image. These results demonstrate that individual NRs have different spectral responses upon changes of the excitation wavelengths. The average spectrum usually referred to is a mix of different spectra from individual NRs. The gradual change in the average spectra (Fig. 4A) is caused by the fraction of NRs with certain spectra (Fig. 4C). For example, under 740 nm or 1000/1040 nm excitations, most NRs show phasors in the long-wavelength region or the short-wavelength region, respectively, which is consistent with the average spectra in Figure 4A. This observation can be obtained only by the spectral phasor method, which builds a connection between spectra and pixels in the spectral imaging analysis.

**Spectral Properties During Optical Trapping**

One interesting observation is the long traces of single NR in the images of Figures 3 and 4, which is obviously due to optical trapping of the NRs. Optical trapping of gold nanoparticles including gold NRs is a potentially important drug delivery method in biomedical applications (Aziz et al., 2011; Gu et al., 2014; Hajizadeh and S.Reihani, 2010; Hansen et al., 2005; Pelton et al., 2006; Selhuber-Unkel et al., 2008). It has been reported previously that gold NRs can be trapped and manipulated by a continuous wave laser beam slightly detuning to the long wavelength side of their longitudinal plasmon resonance (Pelton et al., 2006). Gu et al. further demonstrated that the two-photon absorption of fluorescent nanobeads and gold NRs can enhance the trapping force by 3–4 orders of magnitude (Gu et al., 2014). Interestingly, they reported a snowball effect that is caused by the plasmon-mediated optothermal attracting force over a large range. Therefore, it is interesting to investigate the spectral

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**Fig. 6. Optothermal trapping of NRs** (30 μm above the glass surface) by a high laser power, 15 mW 840 nm, at the center for 3 minutes. (A-B): The intensity image before trapping and corresponding color-coded spectral image. (C-D): The intensity image after trapping and corresponding color-coded spectral image. (E): The phasor plot of B and D. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
properties of NRs during optical trapping and manipulation.

Stock solution of NRs was diluted 10 times, that is, $5.7 \times 10^{10}$ nanoparticles/mL final concentration and followed by 1-hour sonication before measurements. To perform the direct optical trapping, the laser beam at LSPR (840 nm) was focused at 30 μm above the glass surface in the solution and was not scanned during the measurement. However, the pixel clock was still used to trigger EMCCD in order to form a 256 × 256 spectral image. Therefore, X-axis and Y-axis represent the time, with X being the fast time axis. Measurements were taken with 0.512 ms/pixel exposure time and the X line time is ~134 ms. The full spectral imaging was done in 34 s.

In Figure 5, NRs are trapped with an 840 nm femtosecond laser as long as 10s of milliseconds under different laser powers. Image is color coded by corresponding clusters in the spectral phasor plot. The wide distribution of phasors in Figure 5B suggests that a single NR during optical trapping does not have a constant spectrum. Instead, it shows variable spectra that can be identified with spectral phasors as shown in Figures 5A and 5B. Figure 5C shows one trapping trace intensity profile. Different colors in this trace imply the spectral dynamics of NR, which is likely caused by responses of the NR to laser illumination. We also found a stepwise increase and decrease in the intensity in this trace suggesting that one additional NR joined this trap and one NR escaped afterward. This observation is consistent with previous study by Toussaint et al. (2007) who reported that the LSPR of these anisotropic particles can be used to enhance the gradient force of an optical trap and sequential loading of multiple gold bipyramids had been observed.

By increasing the excitation to 5 mW, 10 mW, and 15 mW, more trapping events and longer trapping traces were observed as shown in Figure 5D–5F, in comparison to Figure 5A. It’s worth noting that (1) in the corresponding phasor plots (Figs. 5D–5F), phasors at ~620 nm are obtained, which do not existed in Figure 5B, and (2) the contributions of phasors at ~620 nm increased with higher excitation powers (Fig. 5F). We attribute this shift to heating and melting effects of the individual NRs by the high excitation powers, because it usually happens at the ending stage of the trap traces.

Gu et al. (2014) reported a potentially efficient drug-loading method which is termed as a snowball effect of NRs by a plasmon-mediated optothermal attracting

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**Fig. 7.** Optothermal trapping of NRs (at the glass surface) by a high laser power, 60 mW 840 nm, at the center for 3 minutes. (A–B): The intensity image before trapping and corresponding color-coded spectral image. (C–D): The intensity image after trapping and corresponding color-coded spectral image. (E): The phasor plot of B and D. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
force. They showed the snowball effect as the concentration of gold NR increases to 10^3 per nanoliter with range of up to 4–5 times the radius of the Airy spot. To study this effect, we used the stock concentration of NRs, that is, 5.7 × 10^3 NRs per nanoliter. Raster-scanning images were acquired with 132 × 132 μm^2 area (256 × 256 pixels and 0.512 ms/pixel) before and after the optothermal trapping. Figure 6A shows individual NRs before trapping in solution which are 30 μm above the glass surface and the excitation power was 0.5 mW. The 840-nm femtosecond laser was then parked at the center of the image for 3 minutes with 15 mW average power. Another raster scan spectral image was taken immediately afterward. As a result, Figure 6C clearly shows an accumulation of NRs at the center region of image with a range up to 50 μm in diameter, whereas the focus spot was only ~1 μm during the high power illumination. It proves that this effect is not caused by the direct interaction between laser beam and NRs. As pointed out by Gu et al. (2014), this phenomenon is also fundamentally different from trapping of dielectric microparticles caused by direct heating of water with 170 mW 1.55-μm laser beam (Xin et al., 2011). It is related to localized temperature increase by heating generation from NRs at the focal point with two-photon absorption at their LSPR. Therefore, the optothermal force still exists even with a low-power laser at the water transparent window. The corresponding color-coded images, that is, Figures 6B and 6D, and the phasor plot (Fig. 6E) demonstrate that the majority of NRs trapped have phasors at ~450 nm range and were not altered by 3 minutes 15 mW heating at the focal point, although a few red color-coded NRs are observed in Figure 6D. In summary, this long-range optothermal trapping based on NRs and their LSPR does not change the spectral properties and is a promising drug targeting method and a localized hyperthermia technique for in vivo studies, since the optical trapping in blood stream has already been reported (Zhong et al., 2013).

We also explored the spectral changes of NRs that were trapped at the glass surface as previously discussed by Deng et al. (2012). We moved the focusing plane to the glass surface and performed the spectral measurements. Before the optothermal trapping, immobile NRs with some aggregates were measured in Figure 7A. This area (80 μm × 80 μm) was imaged with 0.512 ms/pixel and 0.5 mW power at 840 nm. Thereafter, the laser beam was parked at the center of the image and its power was increased to 60 mW. After 3 minutes illumination, the same area was imaged again with 0.5 mW excitation power. Surprisingly, (1) we observed a big cluster of gold structure in Figure 7C with ~30 μm diameter, which is far larger than the focal point. A large number of immobile NRs in Figure 7A disappear in Figure 7C, implying the trapping effect covers the whole field of view. This morphology changes and redistribution of NRs are obviously due to the localized heating generated from NRs at the focal point. (2) However, there is no signal detected at the focal point and ring structure was observed, which can be explained by a repulsive optothermal flow and direct laser generated pushing flow at the focal point (Gu et al., 2014; Liu et al., 2010). This phenomenon also exists in Figure 6C, although it is not very obvious due to the free diffusion of NRs after trapping. (3) More interestingly, corresponding spectral phasors and colored images (Figs. 7B, 7D, and 7E) show red shifts after the high-power illumination at the focal point. In Figure 7B, most NRs shows phasors at ~440 nm range with some aggregates that have phasors in the long-wavelength region, possibly due to the interaction between NRs. In Figure 7D, the center area shows spectral phasors at ~620 nm with a broad distribution in the phasor plot (Fig. 7E), which indicates the variance of spectra at pixel level in the spatial image. By color-coding NRs with different spectral phasors (Figs. 7D and 7E), we were able to identify areas with different spectral properties, in complementary to the intensity image shown in Figure 7C. The dramatic change of spectral phasor distribution is attributed to the aggregation of a large number of NRs, although quite a few green color-coded NRs still exist. The spectral phasor information with intensity maps together could help the understanding of the optothermal flow during NR trapping, which is under further investigation. Our results are important in studying interactions between lasers and gold NRs. It is potentially helpful in applications of target drug delivery and thermal therapy.

CONCLUSIONS

In summary, we have demonstrated that the phasor approach can help us to understand the spectral behaviors of NRs in interaction with laser beams at single particle level, which may advance the applications of gold NRs as an effective image and therapy agents. With our high-resolution instrument and the fast spectral phasor analysis, we found NRs with different excitation wavelengths or different powers show different spectra. The wide distributions of spectral phasors in phasor plots together with corresponding spatial images imply that the spectrum from single NR is not uniform, an information that cannot be obtained with typical bulk measurements. The obtained spectra shifts under different imaging conditions are due to fractional changes of NRs with different spectral responses. Clusters of NRs with different spectra can be easily identified in images using the phasor cursors. The phasor approach allows us to investigate the spectral dynamics of NRs during the direct optical trapping and the optothermal trapping. The spectral phasor approach offers a unique tools based on the spectral information to understand the interactions between lasers and gold nanorods. It can be further extended into metal nanoparticles that have unique spectral properties, such as nanocube and others. The obtained complementary spectral information and dynamics could greatly help the study in nanomaterials and their imaging applications in biomedical area.

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REFERENCES


