Three-dimensional harmonic holographic microscopy using nanoparticles as probes for cell imaging

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Abstract: Luminescent markers play a key role in imaging techniques for life science since they provide a contrast mechanism between signal and background. We describe a new type of marker using second harmonic generation (SHG) from noncentrosymmetric BaTiO$_3$ nanocrystals. These nanoparticles are attractive due to their stable, non-saturating and coherent signal with a femtosecond-scale response time and broad flexibility in the choice of excitation wavelength. We obtained monodispersed BaTiO$_3$ nanoparticles in colloidal suspensions by coating the particle surface with amine groups. We characterized the SHG efficiency of 90-nm BaTiO$_3$ particles experimentally and theoretically. Moreover, we use the coherent SHG signal from BaTiO$_3$ nanoparticles for three-dimensional (3D) imaging without scanning. We built a harmonic holographic ($H^2$) microscope which records digital holograms at the second harmonic frequency. For the first time, high-resolution 3D distributions of these SHG markers in mammalian cells are successfully captured and interpreted by the $H^2$ microscope.

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OCIS codes: (090.1995) Digital holography; (160.4236) Nanomaterials; (160.4330) Nonlinear optical materials; (170.6900) Three-dimensional microscopy; (320.7110) Ultrafast nonlinear optics.

References and links

Introduction

Fluorescent bio-markers such as organic dyes [1], green fluorescent protein (GFP) [2], and quantum dots (QDs) [3] have been popularly used for biomedical imaging due to their outstanding brightness and biocompatibility [4, 5]. We have developed a new type of marker using the SHG from noncentrosymmetric nanocrystals which we refer to as "Second
Harmonic Radiation IMaging Probes (SHRIMPs)". Due to the intrinsic properties of SHG, SHRIMPs emit a stable coherent signal which is suitable for long-term observations. These types of observations are usually complicated when using the fluorescent signal because of photobleaching and luminescence blinking [6, 7].

When a nanocrystal of noncentrosymmetric structure is optically excited at a fundamental frequency, it emits the optical signal at the exact doubled frequency. Only materials with crystalline structures lacking a center of symmetry are capable of efficient SHG. As a result, when imaged at the second harmonic frequency, SHRIMPs provide an effective mechanism of contrast between the markers and the generally unstructured or isotropic biological microenvironment. Ordered and highly polarizable biological noncentrosymmetric structures, such as the collagen fibers, have been known for endogenous SHG [8]. However, in most of the biological cell components, the endogenous SHG from the cell interface layers is weak [9].

Compared to fluorescent markers, SHRIMPs allow for significantly better imaging modalities due to their intrinsic properties. Unlike fluorescence, the process of SHG only involves virtual electron energy transition without nonradiative energy loss. Owing to this lossless, virtual transition process, SHRIMPs do not bleach over time and emit a stable, non-saturating signal with a femtosecond-scale response time. This allows for the observation of fast dynamic processes over a long time. Furthermore, SHG is generally a non-resonant process which offers the flexibility in the choice of excitation wavelength. This also results in the flexibility of tuning the wavelength of the SHG signal by changing the excitation wavelength accordingly. The coherent nature of the SHG signal is also a main advantage, providing a possibility to detect the second harmonic signal with interferometric optical techniques [10-13].

The principle of harmonic holography was recently demonstrated [10]. When the digital hologram is recorded at the doubled frequency, it captures both the amplitude and phase information of the SHG object field. Therefore the 3D field distribution, reflecting the 3D distribution of the SHRIMPs could be recorded without scanning. The spatial resolution is limited by the diffraction of light at the half wavelength, and the temporal resolution is limited by the laser pulse duration. Several holographic techniques have been developed for bio-imaging, for example the linear and fluorescent holographic microscopy [14-16]. H² microscopy is especially suitable for long-term imaging with luminescent markers due to the high imaging contrast provided by detecting the signal at SHG frequency: the background signal (linear background scattering and autofluorescence) can be efficiently removed by optical filters.

The SHG properties of several kinds of nanocrystals have been recently reported: ZnO [17, 18], Fe(NO₃)₃ [19], KNbO₃ [20], KTiOPO₄ (KTP) [11, 21] and SHG-active crystalline organic-inorganic hybrid nanoparticles [22]. Reference 22 reported the quantitative characterization of the SHG efficiency from nanocrystals both theoretically and experimentally. In this paper, we show the first quantitative characterization of SHG efficiency from BaTiO₃ nanocrystals. More generally, a theoretical model for the estimation of the absolute SHG power radiated from a single nanocrystal is described. We also report H² bioimaging with BaTiO₃ nanoparticles as SHRIMPs. We demonstrate the H² microscopy where for the first time the 3D distribution of SHRIMPs inside artificial samples and mammalian cells was recorded and reconstructed by one digital hologram.

1. Stabilization of the BaTiO₃ SHRIMPs in a colloidal suspension

The 30-nm and 90-nm BaTiO₃ nanoparticles were obtained commercially in dry powder from Nanoamor and Techpowder respectively. The X-ray diffraction measurement from the suppliers confirmed that the crystal structure is tetragonal, which is non-centrosymmetric, allowing for efficient SHG without any further treatment such as that described in reference 10. The dry BaTiO₃ nanoparticle powder was dispersed in 1.25 mM aminomethylphosphonic acid with the particle concentration of 10¹⁰ particles / cm³. We treated the colloidal suspension.
with ultrasound (Branson digital sonifier 450) for 10 minutes to break the clusters into individual particles. The suspension was then stirred for 24 hours. The phosphonic acid reacts and covers the particle surface [23] and it ends up with an amine group coated particle surface. The suspension is stabilized by electrostatic force from the ionization of the amine group in solution. Finally, we filtered out the big clusters from the suspension by flowing it through a membrane with pore size of 0.22 µm (Millipore Express PLUS). The surface charge of the stabilized particle is determined by the degree of ionization of the amine groups on the particle, which can be tuned by changing the pH of the solution. The zeta potential of the monodispersed colloidal suspension was obtained by light scattering measurement (Brookhaven Instruments Corp. ZetaPALS). We observed that the zeta potential decreases from 10 mV to -40 mV when the pH of the solution increases from 2 to 9. The amine group on the particle also offers many possibilities of crosslinking biomolecules such as proteins, antibodies, and DNAs for specific labeling [24].

We measured the 90-nm particle size distribution in suspension by dynamic light scattering (Brookhaven Instruments Corp. 90Plus) showing that most of the particles were well dispersed while less than 10% of the particles are clusters of double the size (Fig. 1). In the inset of Fig. 1, the scanning electron microscope image confirms the size of the particles which were dried on a conducting substrate.

![Fig. 1. Dynamic light scattering measurement of 90-nm BaTiO₃ particles colloidal suspension. Inset: Scanning electron microscope image of a single 90-nm BaTiO₃ particle.](image)

2. Optical Characterization of the BaTiO₃ SHRIMPs

We measured the SHG efficiency by directly imaging BaTiO₃ nanoparticles. To perform the characterization, we deposited the nanoparticles on a glass substrate. The excitation light source consisted of 150 fs laser pulses centered at 800 nm wavelength (repetition rate 76 MHz) from a Ti:sapphire oscillator. The laser beam was slightly focused to reach the peak excitation intensity on the order of 1 GW/cm² on the sample. The SHG signal was collected by a 0.9 numerical aperture (NA) objective and imaged on an electron multiplying charge coupled device (EMCCD, Andor iXon™ EM + 885). We measured the power dependency and the optical spectrum to confirm the SHG signal (Fig. 2). The SHG signal was observed for an hour with less than 5% decrease under continuous excitation.
We observed the polarization dependent SHG response from isolated nanocrystals by rotating the polarization of the excitation beam. The crystal orientation determines the polarization dependency of the SHG signal [25]. We chose the object with diffraction-limited SHG spot size for the measurement. An artificial frame is placed in Fig. 3 for ease of discussion: the sample plane is in the XY plane, the excitation laser propagates in the Z direction, and θ and φ are defined accordingly in Fig. 3. The direction of the excitation polarization can be described by φ. The measured SHG signals as a function of the angle of the excitation polarization are shown as circular dots in the polar diagram in Fig. 3. We simulated the polarization dependent SHG responses of a BaTiO$_3$ nanocrystal with the c-axis oriented at φ$_0$ = 90 degree and θ$_0$ = 30, 50, 70, 90 degree respectively. Due to the symmetry of the tetragonal crystal structure, the rotation of a BaTiO$_3$ nanocrystal respective to the c-axis does not change the SHG response. In the simulation, the SHG signal was calculated by using the second order susceptibilities of a bulk tetragonal BaTiO$_3$ crystal, where $d_{15} = -41 \times 10^{-9}$ esu, $d_{31} = -43 \times 10^{-9}$ esu, and $d_{33} = -16 \times 10^{-9}$ esu [26]. The simulation model is described in details later in this section. The simulated results show that the orientation of the nanocrystal determines the SHG polar response. When the c-axis tilts (θ$_0$ increases), the polar response changes the shape from a circle to a peanut-shape. When the c-axis rotates in the XY plane (φ$_0$ changes), the peanut-shape polar response rotates. It is worth noting that the orientation (regardless of the direction) of the c-axis of the nanocrystal cannot be uniquely determined from the polar diagram since there are ambiguities when φ = φ$_0$ and φ = π + φ$_0$. The simulated result of φ$_0$ = 90 degree and θ$_0$ = 90 degree matches with the experimental measurement, which implies the orientation of the nanocrystal under analysis is in the XY plane and is aligned in the Y axis direction. The match of the calculation with the measured results also shows that the object under analysis is either a single nanocrystal or a few nanocrystals with the same orientation. When nanocrystals are aligned, they behave the same as one nanocrystal in SHG response.
The absolute total SHG power emitted by the nanocrystal can be found by calibrating the collection efficiency of the optical system. The collection efficiency is the product of the collection ratio of the objective (determined by the solid angle of the numerical aperture), the transmission of the optical components at SHG frequency along the detection, and the quantum efficiency of the detector. The overall collection efficiency in our system was about 1%. Therefore the detected power is 1% of the total radiated SHG power ($P_W$). To describe the brightness of SHRIMPs, we express $W_{2p}$ by as follows:

$$W_{2p} = \sigma_{2p} I_p^2.$$  

where $\sigma_{2p}$ is the two-photon cross section and $I_p$ is the excitation intensity. This has been the widely used expression for describing the brightness of two-photon fluorescent markers. SHG and two-photon fluorescence are both two-photon processes, but the mechanisms of the two processes are very different. SHG is a process without a real state transition. Therefore the $\sigma_{2p}$ of a SHRIMP simply describes how efficient a SHRIMP can scatter at the SHG frequency under excitation at the fundamental frequency. On the other hand, two-photon fluorescence involves a two-photon absorption followed by a fluorescence emission. Therefore the two-photon (action) cross section $\sigma_{2p}$ of two-photon fluorescent markers is the product of the two-photon absorption cross section and the fluorescent quantum efficiency. $\sigma_{2p}$ is measured in unit of Goeppert-Mayer (1 GM = $10^{-50}$ cm$^4$ sec photon$^{-1}$). From the polarization dependency measurement shown in Fig. 3, we obtained a $\sigma_{2p}$ of the isolated BaTiO$_3$ nanoparticle in a range from 2,540 to 19,060 GM. As a reference, the $\sigma_{2p}$ of the commonly used fluorescent bio-markers are listed: 47,000 GM for quantum dots [27], 75 GM for enhanced GFP [28], and 150 GM for Rhodamine 6G [29]. In general, the two-photon fluorescent efficiency depends strongly on the excitation wavelength due to the resonant absorption.

The SHG efficiency of a SHRIMP can be described with a model under the assumption that the SHRIMP is spherical [30]. The size of SHRIMPs in our study is much smaller than the excitation wavelength, so SHRIMPs can be thought as Rayleigh particles. When linearly
polarized electromagnetic waves excite SHRIMPs, the electric field inside the particles is in-phase and uniform as \( E_i(\omega) = \left[ \frac{3\varepsilon_i}{(\varepsilon_i + 2\varepsilon_s)} \right] E_2(\omega) \) \(^{[31]}\). where \( E_i(\omega) \) is the fundamental electric field in the surrounding medium and in absence of the particle, and \( \varepsilon_i \) and \( \varepsilon_s \) are the linear permittivities of the particle and the surrounding medium respectively. The electric field inside the particle will induce polarizations at the fundamental and harmonic frequencies through linear and nonlinear optical susceptibilities \( (\chi^{(1)}, \chi^{(2)}, \ldots) \). The total polarization is then expressed by a power series in the field strength \( E_i(\omega) \) as:

\[
P(\omega, 2\omega, \ldots) = \sum_{n=0}^{\infty} \chi^{(n)} \cdot E_i(\omega) \cdot E_i(\omega) + \cdots.
\]

(2)

The polarization at the doubled frequency gives rise to SHG:

\[
P(2\omega) = \chi^{(2)} \cdot E_i(\omega) \cdot E_i(\omega).
\]

(3)

The \( P(2\omega) \) is thus in-phase and uniform inside the particle and it results in an induced dipole moment of the particle as \( d_i(2\omega) = \frac{4\pi a^3}{3} P(2\omega) \) radiating like an antenna at the SHG frequency, where \( a \) is the radius of the particle. The total emitted power \( W_0 \) is the sum of the power radiated by the three dipole moment components \( d_i(2\omega) \), \( (i = x, y, z) \). We obtain \(^{[32]}\)

\[
W_0 = \frac{c k^4}{12\pi\varepsilon_0} \sum_i [d_i(2\omega)]^2.
\]

(4)

where \( c \) is the speed of light, \( k \) is the wave number at the SHG frequency, and \( \varepsilon_0 \) is the vacuum permittivity. Therefore one can calculate the power of the SHG signal emitted by a SHRIMP under certain excitation intensity. Following equation (1) and (4) with the \( \chi^{(2)} \) of a bulk BaTiO\(_3\) crystal, we can estimate the \( \sigma_{2p} \) of a SHRIMP of certain size, and vice versa. The model indicates that the size of the nanocrystal in the experiment shown in Fig. 3 was 95 nm, which is in the reasonable range as measured by dynamic light scattering. We calculate the \( \sigma_{2p} \) of a 90-nm BaTiO\(_3\) SHRIMP as in a range from 1,735 to 13,010 GM. This range is due to the polarization dependent SHG response. The \( \sigma_{2p} \) is sensitive to the size of the particle, obeying the sixth-power dependency of the \( \sigma_{2p} \) on the radius of the particle. We estimate the \( \sigma_{2p} \) of 30-nm BaTiO\(_3\) particles in a range from 3 to 20 GM under the assumption that the values of \( \chi^{(2)} \) elements remain the same when the particle size decreases. This range is again the result of polarization dependent SHG response.

3. H\(^2\) microscopy

We built a H\(^2\) microscope to demonstrate the potential of SHRIMPs for scan-free 3D imaging. The excitation laser is the same as the one described previously. The peak intensity of the excitation is 1.5 GW/cm\(^2\), which is 100 times less than the cell damage threshold \(^{[33]}\) and is also 100 times less than that used in Ref. 10. The experimental setup is shown in Fig. 4. The H\(^2\) microscope can be understood as a 4F imaging system followed by a holographic recording system. The SHG signal from SHRIMPs was collected and optically magnified 80 times by a 4F system consisting of a 0.9 NA microscope objective and a lens of 20 cm focal length. We put the EMCCD away from the 4F imaging plane so that the object field could propagate and fill the detection area of EMCCD. The hologram recording distance, i.e. the distance between the SHG image formed by the 4F system and the EMCCD, was 20 cm. A plane wave at doubled frequency generated by a separate β-barium borate (BBO) crystal served as the reference beam. The intensity ratio between the signal maximum and the reference beam is set to be 1:10. The signal and reference pulses were collinearly overlapping.
both spatially and temporally on the EMCCD, and as a result an on-axis digital hologram was recorded at the SHG wavelength. The inherent twin image has little effect on reconstructed image due to the long hologram recording distance. The algorithm of holographic reconstruction is described in Ref. 10.

Fig. 4. $H^2$ microscope experimental setup. BS1 and BS2, beam splitters; M, mirror; L1 and L2, lens; S, sample; OBJ, microscope objective; BE, beam expander; F, band-pass filter centered at 400 nm. BS1 splits the laser into signal and reference beams. In the signal arm, L1 slightly focuses the excitation beam into the sample with SHRIMP's. OBJ and L2 form a 4F imaging system to collect and optically magnify the SHG image of SHRIMP. The EMCCD is placed away from the 4F imaging plane. A band-pass filter is placed in front of the EMCCD to remove the excitation from the SHG signal. The reference beam goes through a translation stage and a BBO crystal so that the coherent reference SHG laser pulses are generated and can be temporally and spatially overlapped with the signal on the EMCCD. The signal and reference beams are combined collinearly by BS2 and therefore an on-axis digital hologram is recorded on the EMCCD.

4. Single SHRIMP detection with the $H^2$ microscope

The $H^2$ microscope was sensitive enough to image an isolated $BaTiO_3$ nanocrystal. Since the particle size is smaller than the optical diffraction limit at the SHG wavelength, the image of a single particle reflects the spatial resolution of the $H^2$ microscope. The nanocrystal to be imaged was the same nanocrystal characterized in the polarization dependent measurement as shown in Fig. 3. Figure 5 (a) shows the focused SHG image of the $BaTiO_3$ particle taken by the conventional 4F imaging system. The spot size is 0.4 µm which is the diffraction limit. We then imaged the same particle with the $H^2$ microscope. The single particle behaved as a point source at the SHG frequency. The radiated spherical waves interfered with the reference plane waves and a set of interference rings (Fresnel zone plates) were formed and recorded on the detector plane. By digital propagation, we can reconstruct the field at any plane from the recorded Fresnel zone plates. Figure 5 (b) is the harmonic holographic reconstructed image of the same $BaTiO_3$ particle on the object plane, which shows the same spot size as the Fig. 5 (a). Therefore, the $H^2$ microscope has diffraction-limited resolution. The axial resolution of the $H^2$ microscope can be measured by digital holographic reconstruction of a stack of images along the axial direction. The measured axial resolution was about 2 µm as shown in Fig. 5 (c). The axial resolution may be improved by calibrating the aberration introduced by the optics for the signal collection, especially the spherical aberration introduced by the objective.
5. H² microscopic 3D imaging

The capability of the H² microscope for 3D SHRIMP imaging was first evaluated by imaging an artificial 3D sample. We prepared a 3D distribution of SHRIMPs by randomly embedding 30-nm BaTiO₃ nanoparticles in a ~100-µm thick polydimethylsiloxane (PDMS) film. The SHRIMPs were immobilized inside the film when the PDMS was cured. Figure 6 (a)-(d) show the conventional SHG microscope images of four sub-micron BaTiO₃ clusters on four different planes under excitation. These four clusters were then imaged by the H² microscope. The results are shown in Fig. 6 (e)-(h). We changed the focus by digitally reconstructing the images at different planes. The relative depths of the reconstructed planes were 0, 9.4, 17.2, and 20.3 µm respectively. The reconstructed images in Fig. 6 (e)-(h) agree well with the SHG images in Fig. 6 (a)-(d), suggesting that 3D distributions of SHRIMPs can be reliably recorded and interpreted from a single digital hologram without scanning. It is worth noting that the H² microscopy would have difficulty imaging a high density of SHRIMPs [34]. This difficulty results from the intrinsic speckle noise and it is a fundamental limit of storing 3D information with a 2D image. However, when the density of SHRIMPs is low, the H² microscopy with SHRIMPs makes scan-free 3D imaging possible.
6. 3D imaging of the SHRIMPs in HeLa cells

SHRIMPs make scan-free 3D bio-imaging possible when they are labeled on the targets of interest and imaged with the H² microscope. Here we demonstrate the capability of the H² microscopy with SHRIMPs for 3D bio-imaging, as well as the biocompatibility of BaTiO₃ nanoparticles as SHRIMPs. The sample under analysis was mammalian (HeLa) cells non-specifically labeled with SHRIMPs. HeLa cells were incubated for 24 hours at 37 °C with 30-nm BaTiO₃ particles that have been stabilized with aminomethylphosphonic acid. During the incubation, the amine group present on the SHRIMPs surface encouraged the cells to uptake them through endocytosis [35]. SHRIMPs would be engulfed non-specifically into vesicles and packed as clusters randomly by the cells. In this way a 3D distribution of SHRIMPs was formed inside the cells. Cross section views of a cell imaged by a confocal scanning microscope (Leica, SP5) shown in Fig. 7 confirms that the SHRIMPs entered the cells by endocytosis. After 24-hour incubation, the HeLa cells were still alive.

We washed away the unbound SHRIMPs with a buffer solution (phosphate buffered saline, PBS) and fixed the cells with 3.7% paraformaldehyde. The sample was then ready to be holographically imaged. Figure 8 (a) shows the superposition of the bright field transmission image of a cell and the SHG image from SHRIMPs taken by a conventional microscope. In Fig. 8 (a), we observed some SHRIMPs appeared as bright spots because they were on focus, while the others were not visible because they were out of focus. We assign the SHRIMPs in Fig. 8 (a) with unique numbers for ease of discussion. We imaged the same area with the H² microscope. By digital reconstruction, we observed that several SHRIMPs appeared on different planes. Figure 8 (b)-(d) show the reconstructed images on three different depths of the cell (0, 3.12, and 6.24 µm) where different clusters got on focus respectively. Since the cells uptook the SHRIMPs randomly, the size of the SHRIMP clusters, and therefore the SHG intensity, varied in a wide range (about a factor of 10). We see strong signal from the cluster number 4 both on the depths of 0 and 3.12 µm because it is a big cluster. Figure 8 (e) shows the normalized line intensity profiles of the six clusters when they

Fig. 7. Confocal section images of a calcein-stained HeLa cell (in red) with SHRIMPs (in green) inside it. The excitation consisted of femtosecond laser pulses centered at 800-nm wavelength. Calcein emits two-photon fluorescence signal (in red) while SHRIMPs emit SHG signal (in green). It is clear that the SHRIMPs entered the cell through endocytosis. The scale bar is 5µm.
are on focus through digital reconstruction. All of them show a high signal to noise ratio. The cluster number 3 has a weak signal, and in its line intensity profile we can see the defocused signal from the cluster number 4. The acquisition time of the digital hologram was 1 minute, limited by the integration time for detectable signal. The acquisition time can be further optimized by increasing the excitation power and also the sensitivity of the detection. The peak intensity used in the experiment was 100 times weaker than the critical intensity affecting the cellular metabolism [33], so there is a room to further increase the pump intensity, and, as a result, the SHG signal intensity. The collection efficiency of the SHG signal was ~1%, which could also be significantly improved by adapting the optics for the SHG frequency. Moreover, the reference beam in holography can serve as a coherent local oscillator, leading to a gain in signal to noise ratio and a shot noise-limited performance of detection even at fast read-out [10]. As a pulsed holographic technique, the H² microscopy could reach a temporal resolution limited by the laser pulse width [10, 36].

Fig. 8. Images of SHRIMPs non-specifically labeling HeLa cells (a) Superposition of the bright field transmission image of a HeLa cell (in red) and the SHG image of SHRIMPs (in green) taken by a conventional microscope. Six SHRIMPs clusters assigned with numbers were under analysis. Some of them cannot be seen clearly because they are out of focus. (b)-(d) H² reconstructed images at three different planes with relative depths of 0 µm, 3.12 µm and 6.24 µm respectively. The white arrows show the SHRIMPs that are on focus, while the gray arrows show the SHRIMPs that are out of focus. The SHRIMP labeled with number 4 is a big cluster so that it is bright both in (b) and (c). (e) Normalized intensity line profiles of the six clusters when they are on focus through digital reconstruction. The scale bars are 5 µm.

7. Conclusion

We have demonstrated the H² microscopy with BaTiO₃ nanoparticles as SHRIMPs for high-resolution 3D imaging. The coherent SHG signal from SHRIMPs allows us to capture the 3D distribution of these markers in specimens by a H² microscope without scanning. SHRIMPs possess several unique properties, including long-term observation without photobleaching, flexibility in excitation wavelength, coherent signals for 3D imaging, and ultrafast response time. In general, SHRIMPs are not replacements for fluorescent markers such as GFPs or QDs. Instead they provide new modalities of imaging applications. The BaTiO₃ nanoparticles were stabilized in colloidal suspension by electrostatic repulsion force through ionization of the amine group present on the surface of the particle. A stable suspension of narrow particle
size distribution was obtained. The amine group present on the particle surface also offers the general crosslink for further conjugation to proteins or antibodies for specific labeling. The quantitative polarization dependent SHG response of SHRIMPs was characterized experimentally and theoretically. By dynamically measuring the polarization dependence of the SHG response, we could track the orientation of a SHRIMP. As a result, not only the displacement but also the rotation of a SHRIMP could be monitored in the far field, which completes the detection schemes of SHRIMPs as imaging markers. Taking advantage of the coherent signal of SHRIMPs, we demonstrated the H² microscope as a 3D imaging technique. For the first time, the 3D distributions of SHRIMPs in biological specimens were imaged by the H² microscope with diffraction limited spatial resolution.

Acknowledgments

We are very grateful to Scott E. Fraser and Periklis Pantazis for fruitful discussions on the SHG efficiency characterization. We are also grateful to Stephen Quake, Stavros Stavrakis, and Jerrod Schwartz for helpful discussions on the colloidal stabilization. We thank Paul Bowen for providing the BaTiO₃ nanoparticles, Robin Humphry-Baker for the assistance with the light scattering measurement, and Marc Chambon for the help of HeLa cells preparation. The author C.-L. H. would like to thank Taiwan Merit Scholarship (TMS-094-2-A-042) for financial support. The author Y. P. would like to thank the Optofluidic Center for financial support. This project is supported by National Center of Competence in Research (NCCR), Quantum Photonics.