Digital holography for second harmonic microscopy

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ABSTRACT

Quantitative phase images make digital holographic microscopy (DHM) an excellent instrument for metrological, but also for biological applications, where it can reveal deformations and morphological details at ultrahigh resolution in the order of a few nanometers only, while also precisely determining the refractive index across a sample (e.g. cell or neuron). On the other hand, non-linear light-matter interactions have also proved very useful in microscopy. For instance, second harmonic generation (SHG) allows marker-free identification of cell structures, tubulin or membranes and, because of its coherent nature, SHG is very sensitive to the local sample structure and to the direction of the laser polarization. In addition, since SHG does not result from light absorption and subsequent re-emission, in opposition to fluorescence, photo-bleaching of the studied material can be avoided by a judicious selection of the laser wavelength. These characteristics make SHG very interesting for biomedical imaging. We have designed and built a microscope that combines the fast and precise DHM imaging with tagging capabilities of non-linear light-matter interactions. Here, we present the technique and look into its possible applications to biological and life sciences. Among promising applications is the 3D tracking of colloidal gold nanoparticles.

Keywords: digital holography, second harmonic generation, gold nanoparticles

1. INTRODUCTION

With the downscaling of material sciences to the submicrometer range, nanomaterials of all sorts have attracted a lot of interest and are now being used for numerous applications in a very large range of research fields and industrial activities. Nanoparticles (NPs), in general, are extensively used in biology as labels or contrast agents for imaging or detection of pathogens (or proteins) and functional monitoring of biological processes. In addition, they form the building blocks of bottom-up tissue engineering, and drug or gene delivery to specific cellular sites and even for tumor treatment. Gold NPs, in particular, appear very promising, thanks to their excellent biocompatibility and their chemically inert nature. As a consequence, very good knowledge of gold surface biochemical functionalization has been developed [1] and gold NPs now constitute an excellent base for bio-conjugated probes.

After functionalization, gold NPs become highly selective and will chemically bind only to the targeted protein – they are said to be bio-conjugated. However, even though biochemical selectivity is very high, actual optical detection and monitoring techniques (fluorescence [2], enhanced scattering [3], nonlinear microscopy [4]) do not provide comparable spatial resolution, being at best diffraction limited in the lateral direction and no better in the axial direction. Among the better resolved technique is second harmonic generation (SHG) microscopy. In this work, we propose a holographic method that allows complex SHG wavefront retrieval and therefore provides real-time 3D tracking of gold NPs.

2. DIGITAL HOLOGRAPHY FOR SECOND HARMONIC MICROSCOPY

Holography consists in using a reference wave to encode the complex diffraction pattern of an object into an interference image called hologram. Of course, this definition assumes that the two (reference and object) waves have mutual coherence properties. Classically, holograms were recorded on a photosensitive plate and had to be photo-chemically developed and re-illuminated to produce seemingly 3D images. For the special case of digital holography (DH), holograms are recorded with a digital camera and image reconstruction is numerically performed by a computer. It is not the scope of this work to elaborate on the basis of digital holography. Details of the technique, as well as its application to non-linear fields can be found in Ref.[5].

The main advantage of DH is that reconstructed images contain information on both the amplitude and the phase of the object wave, which makes possible measurement of nanometer-scale surface topography [6] and mapping of refractive index distributions with a very high precision (10^{-3} and better) [7]. This explains why DH exceeds in both metrology and biology.

Digital focusing is another advantage of the technique. Because DH recovers the complex object wavefront, diffraction-based wave propagation equations can be used to numerically propagate the image field. This feature lifts any constraints on the camera position relative to the system image plane, and holograms can be recorded out of focus and images numerically reconstructed and focused, as depicted in Fig.1. Another, even more important consequence of this is the extended depth of focus of digital holographic microscopy, compared to other microscopy techniques, as objects lying outside the depth of focus of the microscope objective can still be reconstructed and numerically propagated to their respective image plane, and therefore be brought into focus.

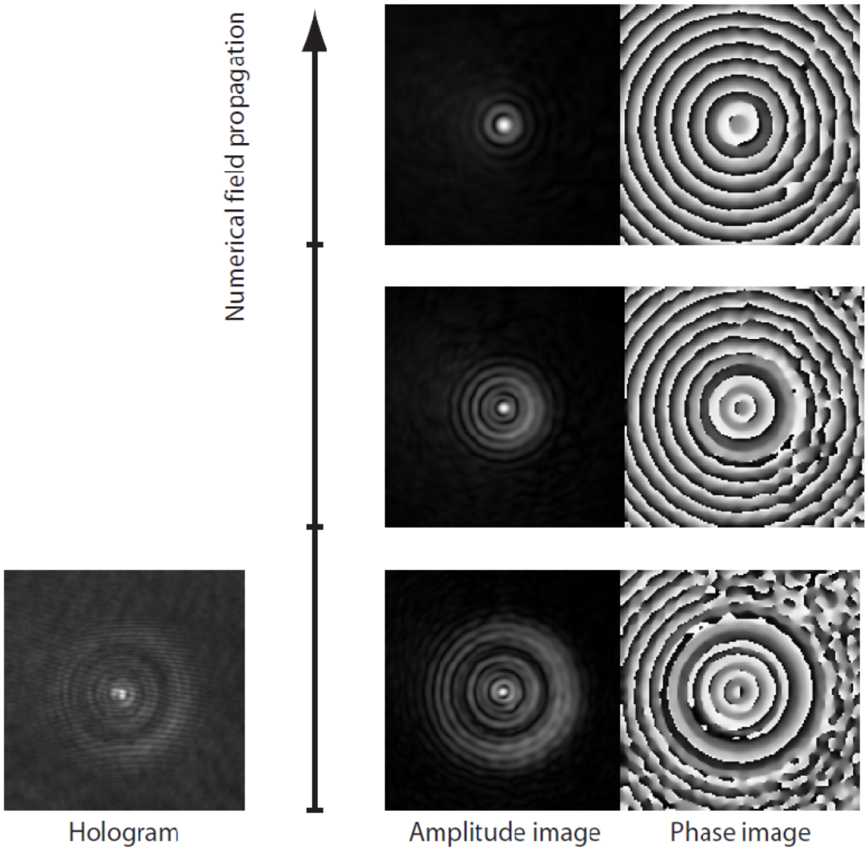

Figure 1. Illustration of digital focusing. The hologram (left) is recorded out of focus and the recovered complex field (right, bottom-most amplitude and phase images) is digitally focused by numerical propagation algorithms to the image plane (right, top-most image amplitude and phase images).

Furthermore, and again because it recovers the complex field, DH has a better sampling of weak signals than any intensity-based imaging technique. Fig.2 compares amplitude and intensity signals of a second harmonic emitter – from a specimen consisting of gold NPs. The dynamic range of the weaker signals is evidently higher for an amplitude-based technique. In addition, hologram can be recorded out of focus to spread the signal from a strong scatterer on many pixels, to avoid saturating the camera or compromising the dynamic range of weaker signals. Moreover, and thanks to its interferometric nature, DH benefits from coherent amplification [8]. This means that for a given number of photons in the object wavefront, the signal to noise ratio can be significantly increased, even doubled, simply by increasing the number of photons in the reference wavefront. This is extremely important for nonlinear signals, like SHG, that are generally of relatively low intensities.

Finally, off-axis DH is a full-field, single shot acquisition technique. Since it requires no scanning whatsoever, it is very fast (real-time acquisition and processing) and insensitive to vibrations, for relatively small camera shutter times.

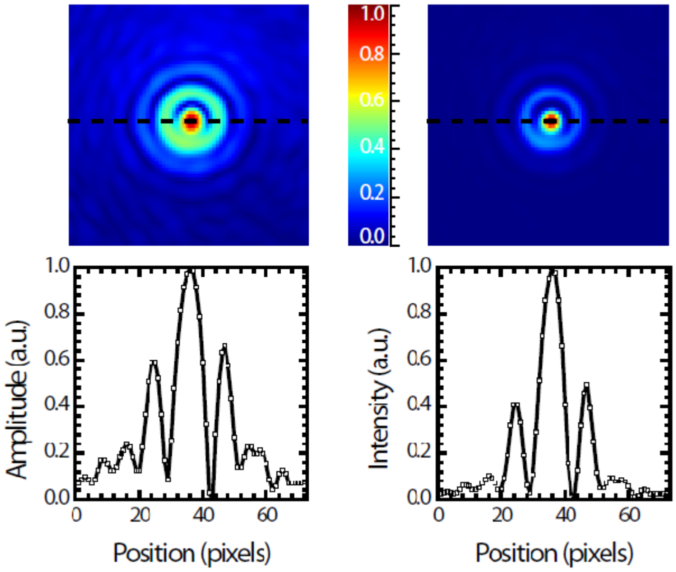

Figure 2. This amplitude vs intensity comparison of the second harmonic signal generated by a single nanoparticle illustrates that an amplitude-based imaging technique provides a better dynamic sampling of weaker signals.

3. 3D LOCALIZATION OF SECOND HARMONIC EMITTERS

Schematics of a typical second harmonic digital holography setup can be found in Fig.3. Light source is a femtosecond laser equiped with a regenerative amplifier stage that delivers 5 µJ, 250 fs pulses at a repetition rate of approximately 250 kHz. The Mach-Zehnder-type interferometer consists of one object arm containing the specimen and one reference arm for the reference wave. In the object arm, light is focalized in the specimen plane to a spot size of about 30 µm and collected by a 100X microscope objective, therefore allowing full-field imaging. A frequency doubler β-barium borate crystal is inserted in the reference arm to generate the second harmonic reference wave. Holograms were recorded using a −20 °C-cooled Hamamatsu Orca-ER CCD camera. A 400 ± 20 nm bandpass filter was used to isolate the SHG signal from the fundamental.

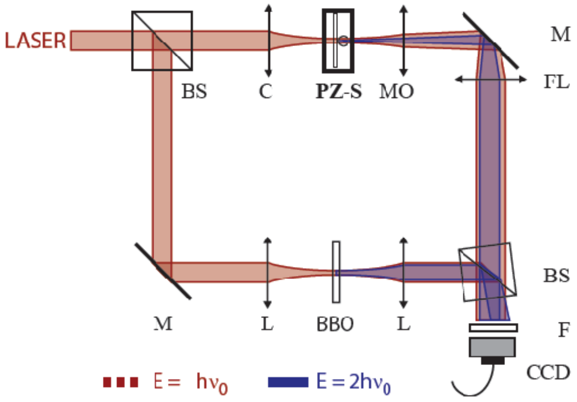

Figure 3. Simplified experimental setup schematics. LASER, 250 fs Ti:Sapphire laser; BS, beam splitter; C, condenser lens; PZ-S, piezoelectric scanner mounted specimen; MO, 100X microscope objective; M, mirror; FL, field lens; F, wavelength selective filter; L, lens; BBO, frequency-doubler Beta-barium borate crystal and CCD, digital CCD camera.

A solution of 60 nm diameter colloidal gold NPs was deposited on a glass coverslip and let to dry for several hours before being imaged in second harmonic microscopy with our digital holographic microscope. For comparison, Fig.4 displays a SHG intensity image (Fig.4a) as well as the amplitude and phase image representation of the complex SHG field obtained by digital holography. The intensity image shows what any intensity-based imaging technique would produce for the same setup characteristics (field of view, NA, etc.) for a camera located in the system image plane. However, since the camera was located at some distance from the system image plane, the complex SHG field recovered from direct processing of the hologram is completely out of focus (amplitude and phase image representations in Fig.4b and Fig.4d) and has to be digitally focused to be compared with Fig.4a. After digital focusing, one obtains the amplitude and phase image representations of Fig.4c and Fig.4e. While the lateral position of the SHG emitters is straightforwardly determined by any of the above-mentioned images, no conclusive information on their axial position can be obtained from an intensity image like Fig.4a and only access to the complex SHG field can provide information on the axial position of the SHG emitters.

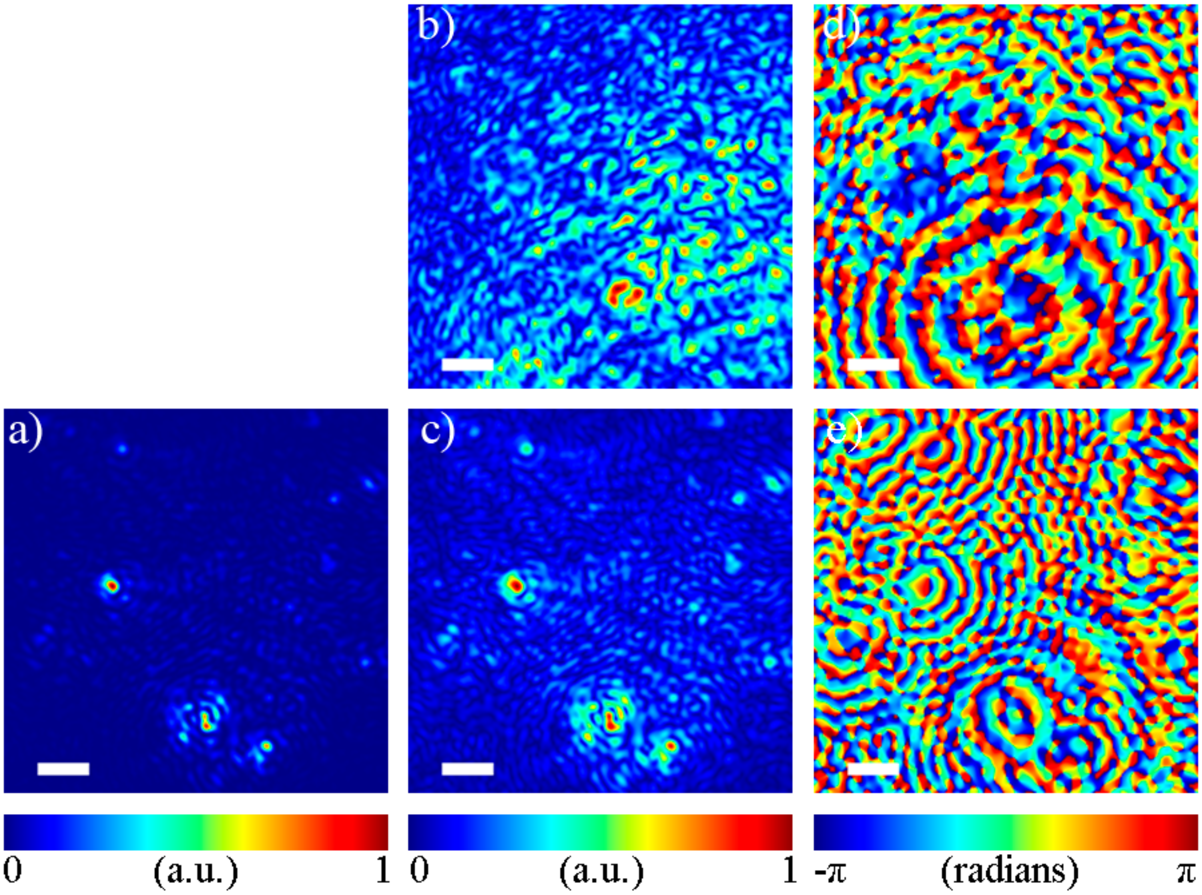

Figure 4. Second harmonic generated by gold nanoparticles deposited on a glass coverslip: a) intensity image, (b) and (d) completely out of focus amplitude and phase images of the complex second harmonic field recovered by digital holography, (c) and (e) amplitude and phase images of the complex second harmonic field recovered by digital holography, after digital focusing. Scale bars are 2 microns.

One way of finding the axial position of the SHG emitters is by using digital focusing. Since digital focusing is based on wave propagation equations, the input parameter that determines the extent of the focusing is the propagation distance, in the image space. In simple terms, the correct focusing is obtained for a numerical propagation of the field over a distance that corresponds to the distance between the camera that recorded the hologram and the system image plane. For point-source emitter objects, the image will be most intense for that optimal propagation distance. If two objects, or here SHG emitters, are at a slightly different axial position in the object space, they would be imaged at two different axial positions in the system image space, and in terms of digital focusing, they will require the propagation distance to be correspondingly different to produce a sharp, in focus image. Fig.5 shows axial cross-sections of the SHG intensity for two different emitters, located at different axial positions. Cross-sections are obtained by varying the field propagation distance in the image space over a few centimeters. This translation of the image plane can be related to an axial translation of 2 microns in the object space. The graph in Fig.5 plots the normalized intensity of the SHG field as a function of the axial object space coordinates, for two different SHG emitters: A and B.

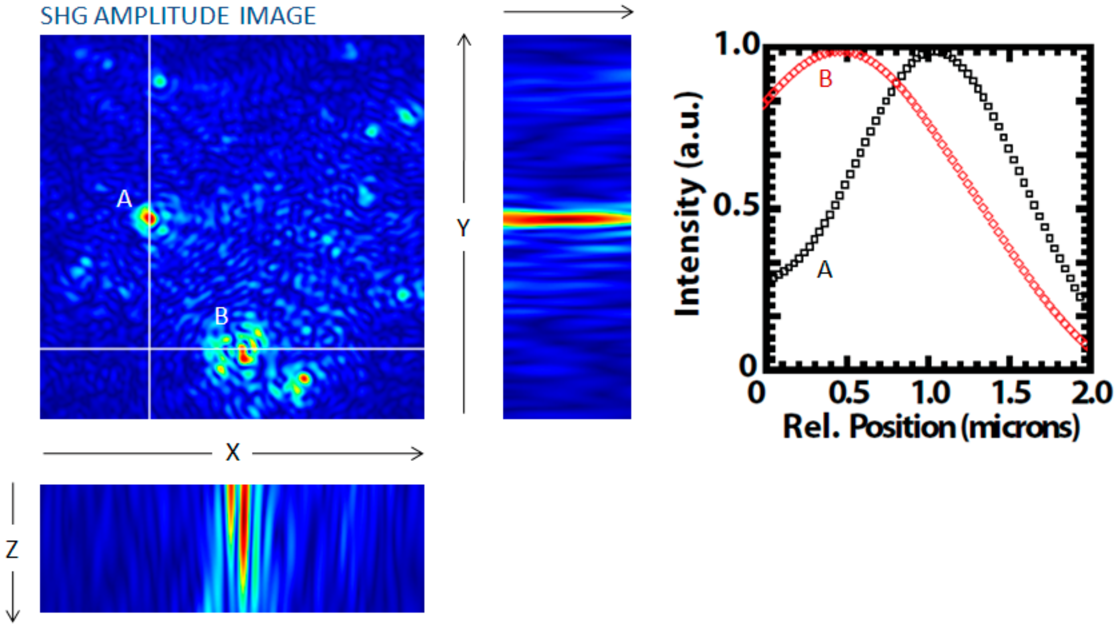

Figure 5. XZ and YZ second harmonic intensity cross-sections (left) obtained by varying the propagation distance in the digital focusing algorithm. The graph (right) plots the intensity of two different emitters A and B as a function of the object-space axial coordinate Z corresponding to the variation in the propagation distance, in the image space coordinates. The relative axial position of the two emitters can be directly deduced from the graph.

4. CONCLUSION

In conclusion, we used digital holography to recover the complex fields from a non-linear light-matter interaction, namely second harmonic generation. More importantly, we have shown that the retrieval of the complex field provides information that cannot be accessed by other, non-interferometric techniques. In this specific case, the phase of the second harmonic generated by nanoparticles provides direct, nanometric information on their relative axial position, which makes possible their real-time, 3D tracking.

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