Background-free 3D four-wave mixing microscopy of single gold nanoparticles inside biological systems

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Abstract: Individual small gold nanoparticles are imaged in 3D background-free with high contrast by four-wave-mixing interferometry inside living mouse oocytes and multi-cellular organs, despite the strong linear scattering background in these large samples.

1. Summary

The development of optical microscopy technologies to study biological cells with increased sensitivity, specificity, spatial resolution and imaging speed continues to be a major drive. In parallel to this development, there has been huge progress in manufacturing nanoparticles (NPs) tailored to provide a combination of optical, chemical, thermal, magnetic, and/or mechanical properties with diagnostic and therapeutic capabilities for biomedical applications. In this context, gold nanoparticles (AuNPs) are being widely investigated owing to their small sizes, bio-compatibility, chemical stability, facile surface chemistry for bio-conjugation, and strong photothermal properties. Notably, AuNPs exhibit a strong and wavelength-selective optical absorption and scattering at the localised surface plasmon resonance (LSPR) which provides a means to visualise them by optical microscopy. However, techniques in the linear light-matter interaction regime, such as bright-field extinction, dark-field, interferometric scattering, and differential interference contrast (DIC) are not background-free. Indeed, it is challenging with these methods to distinguish a single small AuNP against the endogenous scattering, absorption and phase contrast in a highly heterogeneous three-dimensional (3D) cellular environment.

Recently, we have introduced a nonlinear microscopy method based on four-wave mixing (FWM) interferometry triply-resonant to the LSPR, as a very selective, background-free, photostable method to detect single small AuNPs inside cells, with high signal-to-noise ratio at useful imaging speeds and low excitation intensities, compatible with live cell imaging [1–4]. Importantly, the method offers a very high spatial resolution and sectioning capabilities, providing an accurate location of AuNPs inside complex samples in 3D. While our previous works demonstrated the technique on 2D cell cultures, here, we show imaging of single small AuNPs inside large 3D cellular and multi-cellular volumes.

A sketch of the FWM microscope is shown in Fig. 1a, alongside a demonstration of time-resolved FWM detection (Fig. 1b) and FWM imaging (Fig. 1c-f). FWM excitation and detection in this set-up can be understood as a pump-probe scheme [2]. Optical pulses of 150 fs duration centered at 550 nm wavelength with $\nu_L = 80$ MHz repetition rate are split into three beams (pump, probe and reference) having the same center optical frequency. The pump beam excites the AuNP at the LSPR, with an intensity $E_1 E_1^*$ that is modulated at $\nu_m = 0.4$ MHz by an acousto-optic modulator (AOM). The change in the AuNP optical extinction (see sketch in Fig. 1b) induced by this excitation is resonantly probed by a probe pulse of field E_2 at an adjustable delay time τ after the pump pulse. Pump and probe pulses are recombined into the same spatial mode and focused onto the sample by a high numerical aperture (NA) microscope objective (MO). The sample is positioned and moved with respect to the focal volume of the objective by scanning an xyz sample stage. A FWM field (proportional to $E_1 E_1^* E_2$) is collected by the same objective (epi-detection), together with the probe reflected field, transmitted by an 80:20 (T:R) beam splitter (BS1) used to couple the incident beams into the microscope, and recombined in a 50:50 beam splitter (BS2) with a reference pulse field of adjustable delay. The resulting interference is detected by two pairs of balanced photodiodes (BP). A heterodyne scheme discriminates the FWM field from pump and probe pulses and detects the amplitude and phase of the field [3]. In this scheme, the frequency of E_2 is up-shifted by $\nu_2 = 82$ MHz, and the FWM field at the corresponding beat radiofrequency $\nu_2 - \nu_L \pm \nu_m$ is detected by a lock-in amplifier, separated from E_1 and E_2 and thus free from scattering background. We use a multi-channel lock-in which enables the

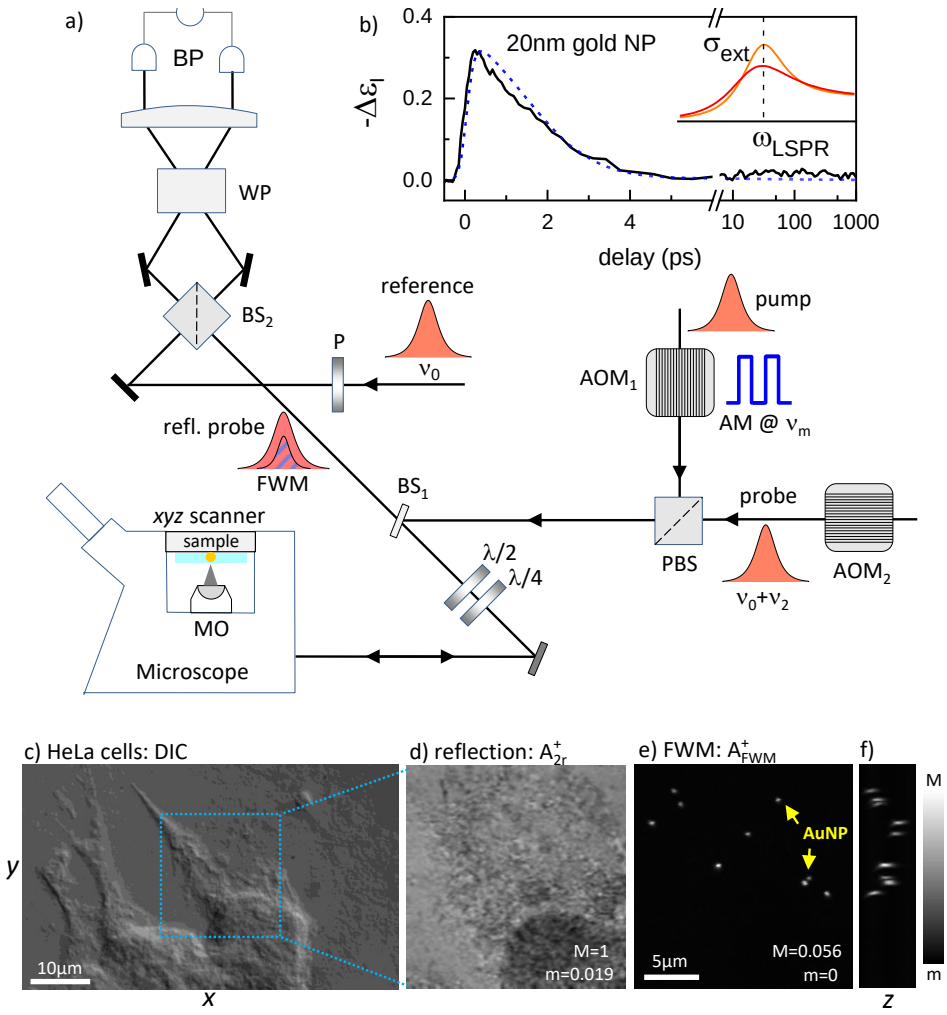

Fig. 1. a) Sketch of the FWM microscope set-up (see text); b) example of transient FWM in a single AuNP of 20nm diameter. Black solid curves: Measurements. Blue dashed lines: Simulations following our model in [2]. c-f) FWM imaging in fixed HeLa cells that have internalized 40 nm diameter AuNPs (see text and [4]).

simultaneous detection of the reflected probe, at the carrier frequency $\nu_2 - \nu_L = 2$ MHz, and of the sidebands at $\nu_2 - \nu_L \pm \nu_m = 2 \pm 0.4$ MHz. Notably, the interferometric detection is also insensitive to incoherent fluorescence backgrounds. Moreover, by varying τ, the electron dynamics can be time-resolved (see Fig. 1b), revealing the time scale of the thermalisation between electrons and the lattice of the metal (~ 1 ps) and the subsequent thermalisation of the NP with the surrounding medium (> 100 ps). This delay time dependence enables us to distinguish the electronic NP response from instantaneous coherent backgrounds and/or long-lived photothermal effects. As a result, we can achieve background-free FWM imaging of single small gold NPs even in scattering and fluorescing cellular environments. An example of high-resolution FWM microscopy (using a 1.45NA oil immersion objective) is shown in Fig. 1c-f on fixed HeLa cells that have internalised AuNPs of 40 nm diameter. Fig. 1c shows the DIC image of a group of HeLa cells on which reflection and FWM imaging (at $\tau = 0.5$ ps) was performed in the region highlighted by the dashed frame. The reflection image (amplitude of the reflected probe field) shown in Fig. 1d correlates with the cell contour seen in DIC. Even with a particle diameter as large as 40 nm, AuNPs are not distinguished from the cellular contrast in DIC or in the reflection image. On the contrary, the FWM field amplitude shown in Fig. 1e-f as a maximum intensity projection in the xy and yz planes, over a 5 μm z-stack, is background-free and clearly indicates the location of single AuNPs in the cell. Note that the set-up also features a dual circular-polarization detection scheme, using a $\lambda/4$ and $\lambda/2$ wave-plate to adjust the circular polarisation of the pump and probe fields (see Fig. 1a), a reference field linearly polarised at 45 degrees, and a Wollaston prism (WP) which separates the horizontal and vertical polarizations for each arm of the interferometer after BS2 [3]. Data shown here refer to detecting the co-circularly polarised components of the reflected probe field and FWM field relative to the incident circularly polarized probe, having amplitudes indicated as A^+_{2r} and A^+_{FWM} respectively.

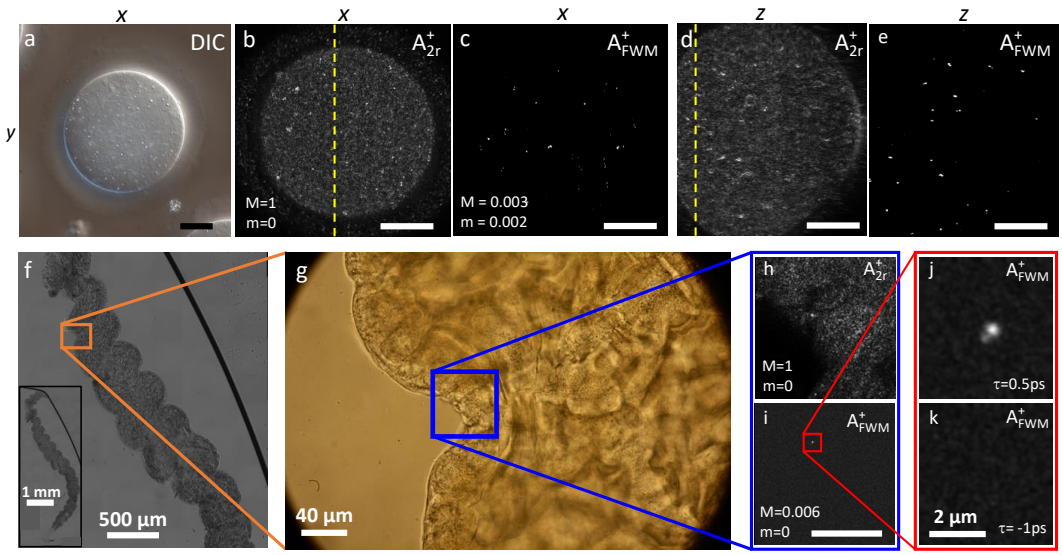

Fig. 2. a-e) Living mouse oocyte micro-injected with AuNPs of 30 nm diameter (see text). f-k) Hepatopancreas of *O. asellus* exposed to food spiked with 20 nm diameter AuNPs (see text). Scale bars: 20 μm unless otherwise indicated.

Going beyond 2D cell cultures, we have investigated the applicability of the technique to locate single small AuNPs inside large 3D volumes. Fig. 2a-e show a living mouse oocyte that was micro-injected with AuNPs of 30 nm diameter. Fig. 2a is a high resolution DIC (using a 60× 1.27 NA water immersion objective), while Fig. 2b-e are the corresponding reflectometry and FWM images, using a pump (probe) power at the sample of 40 μW (20 μW), a pixel size of 0.07 μm in x,y and 0.5 μm in z, and a pixel dwell time of 0.25 ms. Oocytes are large round cells of nearly 100 μm diameter. Fig. 2b,d show reflection images as xy and yz planes (taken at the indicated yellow lines). Fig. 2c,e are the corresponding FWM images where individual AuNPs are visualised and resolved over such large volume. Another example of FWM imaging inside a large multi-cellular system is given in Fig. 2f-k, where the hepatopancreas (HP) of the terrestrial isopod *Oniscus asellus* (the common woodlouse) is shown. This isopod can ingest metal NPs from the environment and its HP plays an important role, being involved in metal resistance/tolerance. Here, we investigated the HP from adult organisms exposed to food spiked with 20 nm diameter AuNPs. Fig. 2f,g are bright field transmission images of regions of the HP, which is a very large multi-tubular organ, a few millimetres in length. Fig. 2h is a reflection image of the region highlighted in Fig. 2g and Fig. 2i is the corresponding FWM image (using similar excitation and detection conditions as for the oocyte). A further zoom is shown in Fig. 2j,k for $\tau = 0.5$ ps (j) and $\tau = -1$ ps (k), to exemplify the transient behaviour of the measured signal as expected for FWM resonant with the LSPR of AuNPs. Work is in progress to correlate the location of AuNPs with two types of cells forming the HP of *O. asellus* and playing a different metal sequestering role. Notably, due to the wide range of industrial applications with metal NPs, high amounts of them are entering ecosystems. Thus, beyond proof of principle, this work will be important to understand the fate of metallic NPs in terrestrial isopods as model organisms in soil ecotoxicology.

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