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3D Nanometer Images of Biological Fibers by Directed Motion of Gold Nanoparticles

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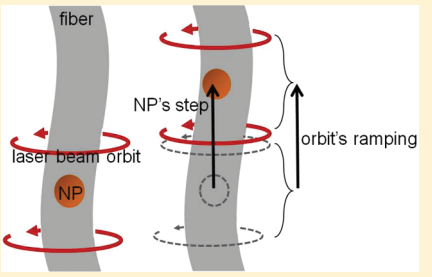
Supporting Information

ABSTRACT: Using near-infrared femtosecond pulses, we move single gold nanoparticles (AuNPs) along biological fibers, such as collagen and actin filaments. While the AuNP is sliding on the fiber, its trajectory is measured in three dimensions (3D) with nanometer resolution providing a high-resolution image of the fiber. Here, we systematically moved a single AuNP along nanometer-size collagen fibers and actin filament inside chinese hamster ovary K1 living cells, mapping their 3D topography with high fidelity.

KEYWORDS: Plasmonics, gold nanoparticles, single particle tracking, photoacoustic, two-photon excitation



Advances in scanning probe microscopies (SPM) allow very detailed measurements of nanometer-size features in a variety of conditions. However, none of the current SPM techniques seems capable of measuring the nanostructure of filaments (such as F-actin) in real time, in three dimensions (3D) and inside living cells. Here, we described an optical method to move, in a controlled manner, single gold nanoparticles (AuNPs) in 3D along fibers and inside live cells, using a two-photon excitation microscope (Figure 1a). The gold enhancement nano-imaging (GENI) method introduced in this work is based on the photoacoustic effect (PA) at the fiber position caused by the irradiation with ultrashort, near-infrared laser pulses as was previously described by our group.[1] The PA effect allows us to directionally move a single AuNP when it is adsorbed to the surface of a fiber. While the AuNP is sliding, its trajectory follows the 3D structure of the underlying fiber. Briefly, when fibers are irradiated with ultrashort-laser pulses, as in a two-photon excitation microscope, there is rapid local thermo-elastic expansion of the surrounding medium due to energy conversion from light to heat, which induces a pressure wave propagating away from the point of energy deposition. The driving force for AuNPs stepping along the fiber is due to the near-infrared absorption of the excitation light that couples to the surrounding solvent. The pressure wave generated is sufficient to move by a certain step (in the tens of nanometers range depending on the incident power, the excitation wavelength, and the irradiation time) the AuNPs each time the laser is absorbed in the volume near the location of the AuNP.[1] According to this mechanism, the NP to be moved must be attached to a fiber, and the fiber provides the direction of the motion.

To apply the GENI method, the computer routine starts with a 2D fluorescence image of the sample. Usually (but not necessary) this image is taken in the *xy* plane. Only in samples incubated with AuNPs, diffraction-limited bright spots of width $\sim\lambda/(2\mathrm{NA})$, where λ is the excitation wavelength and NA is the numerical aperture of the microscope objective, are clearly seen. To confirm that the bright spots are single AuNPs, we reconstructed at the spot location the plasmon absorption spectra exciting the sample with 457.9, 488, 514.5, 543.5, and 632.8 nm in a commercial confocal microscope (Olympus FV1000, Japan). The fact that the position and the shape of the plasmon absorption of metal particles are strongly dependent on the shape,[2−5] size,[3,6,7] and particle material allows us to identify the bright spots present in the images with the spherical single AuNPs of 20 nm in diameter.

To control the NP's movement, we used a two-photon excitation microscope. First we obtain a raster scan image and identify the diffraction-limited bright spots corresponding to the AuNPs. Then we select the NP to be moved by positioning the femtosecond laser diffraction-limited beam on the center of the bright spot. A detail description of the instrument used for these experiments is available in the Instrumentation Section in Supporting Information. The microscope scanning mirrors and the piezo device are controlled by an ISS 3-axis card (version 1.0, ISS, Champaign, IL). In the following we describe our method to track the position of the particles and to push the particle along the fiber. Tracking is performed using the orbital tracking method previously described.[15] Briefly, the combination of scanning mirrors and/or the piezo are driven by a sine and cosine function, respectively, in order to obtain a circular orbit of a diameter of about 300 nm at an arbitrarily oriented plane. The center of the orbit is moved to follow the center of mass of the intensity measured along the orbit.

Clicking at any point in the image directs the laser beam to the chosen point by changing the DC offset values. During each tracking cycle, the excitation beam traces *n* circular orbits in a plane at one side of the particle and *n* orbits in a plane on the other side in the direction of the fiber. This is also controlled by

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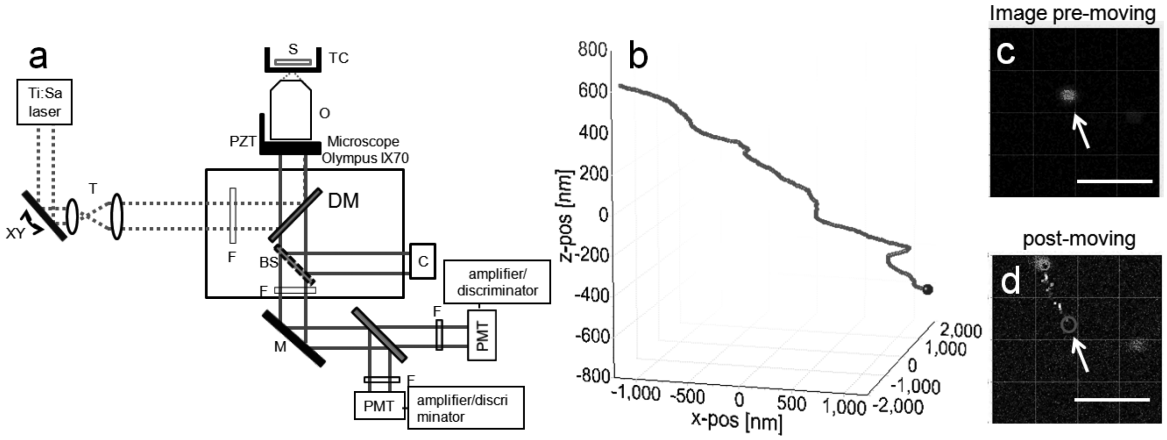

Figure 1. GENI method. (a) Schematic representation of the two-photon microscope used for these experiments, where XY = galvano scanners, T = telescope, F = filters, BS = beam splitter, DM = dichroic mirror, PZT = piezo-driven objective, O = microscope objective, S = sample, TC = temperature control device, M = mirror, PMT = photomultipliers, and C = camera. (b) 3D trajectory reconstruction of a single 20 nm AuNP moving along a collagen type I rat tail fiber. Each point in the trajectory represents the position of the AuNP during one cycle of tracking. The blue dot represents the initial tracking position. (c) Fluorescence image before the AuNP movement. Scale bar is 3.5 μm. (d) Fluorescence image after the AuNP movement. The red circle shows the initial AuNP's position, the blue circle the final AuNP's position, and the dots the 2D trajectory projection. Scale bar is 3.5 μm.

the ISS 3-axis card which generates a square wave voltage signal to drive the motion of the corresponding axis between two planes. The separation distance between planes is given by the amplitude of the square wave. If the NP moves from the center of the orbit, then the detector measures a change of the collected intensity along the orbit. From this data, we determine the angle and the distance from the center of the orbit, which allows the calculation of the new position of the NP. When the NP's new position is known, the orbit's position is rapidly changed (in the millisecond range limited by the time response of the galvo and piezo drivers). This process keeps the NP always centered with respect to the orbit. During each cycle of the tracking routine, two orbits of 256 points and a dwell time of 32 μs were used to measure the intensity signal. After one cycle of the tracking routine, we displace the orbit a predefined distance (in the nm range) in a direction along the fiber (see Figure S1 in Supporting Information). As the orbit moves along the fiber, the laser first "kicks" the NP, and then the algorithm "tracks" (with nanometer resolution limited by the number of collected photons) the new NP's position. Since the NP is electrostatically attached to the fiber, the NP's step can only occur along the fiber itself. To maintain the consecutive kicking of the NP, the orbit's displacement should compensate the NP's step during one kick which depends on the NP's size and shape, laser's wavelength and power, and the laser's irradiation time. In other words, during the orbit movement along the fiber, the corresponding axis is ramped continuously, while the transversal orbit's position changes according to the feedback tracking mechanism following the topography of the fiber. In summary, we can separate the GENI method introduced in this work in two parts: a tracking and a "kicking" part. Due to the time response of our current piezo-scanner device, in a typical experiment, the period of the movement in the z-axis is ~30 ms, whereas in the x and y axis is much faster (in the millisecond range).

To test the GENI method, we have first measured single collagen fibers (Figure 1b–d). Understanding both the structural and functional properties of collagen type I fibers is of special interest to researchers because the structural modifications of the fibrillar matrix are associated with physiologic processes, such as diabetes, aging, wound healing, and cancer.[8−10] Also, changes in the collagen nanostructure have been used to identify breast tissue malignancy.[11] In order to find a AuNP attached to a collagen fiber, we first took a raster scan fluorescence image using two acquisition channels: one channel was set up for the second harmonic generation (SHG) of collagen fibers and the other channel for the signal from the AuNPs. Each point in Figure 1b represents the position of the AuNP during one cycle of tracking (16 ms). For this study, we used 20 nm AuNPs (BBInternational, Cardiff, U.K.) and collagen type I rat tail hydrogels samples (BD Biosciences, Bedford, MA). Once a AuNP attached to a fiber is found, we proceeded as explained in the Supporting Information section. The orbits during the GENI approach can be performed in any Cartesian plane; however, as shown in the raster scanning image, the fibers are mostly aligned along the y-axis. In Figure 1b–d we performed a circular scanning in the xz-plane which will produce a NP movement mainly in the y-direction (always confined along the fiber). Then, the orbit along the xz-plane is slowly moved along the y-direction. The movement in the y-direction was set between 1 and 10 μm, at a mean velocity ~28 nm/sec. The 3D trajectory of a single AuNP along the collagen fiber is shown in Figure 1b. The fluorescence images taken pre- and post-NP moving show very clearly an ~4 μm NP's displacement (Figure 1c and d). Notice the colocalization between the final positions of the orbit's (small circle) and the NP's (bright spot) which confirms that the NP was effectively moved during the GENI experiment (Figure 1d). The GENI method allows the particle to freely move in the orbit–plane to follow the local topography of the collagen fiber while it is being "kicked" along the fiber. As was already discussed, the orbit's plane and consequently the ramp direction can be arbitrarily chosen according to the main fiber's orientation. In order to characterize the AuNP's emission process, we performed a two-photon excitation test at the AuNP's position (see Figure S2 in Supporting Information). The slope of the excitation power dependence of the emitted signal in a log–log plot resulted to be 2.46 ± 0.07, showing a multiphoton emission process.

To demonstrate the reproducibility of the method, we slowly (in the ~nm/s range) moved a single AuNP several times back

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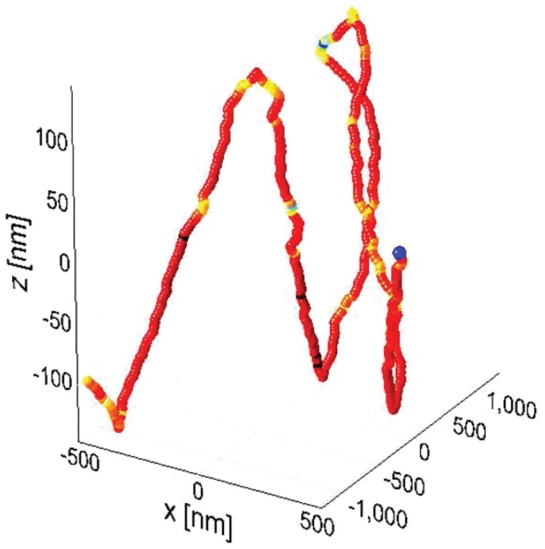

Figure 2. 3D GENI trajectory reconstruction moving the AuNP back and forth. 3D trajectory for a 20 nm AuNP moving along a label-free collagen fiber. Each point represents the position of the AuNP during one cycle of the tracking, and the colors changes with the collected intensity. The blue dot indicates the beginning of the trajectory. All the dimensions are in nm. Note that during the experiment the fiber moves within the diffraction-limited microscope resolution.

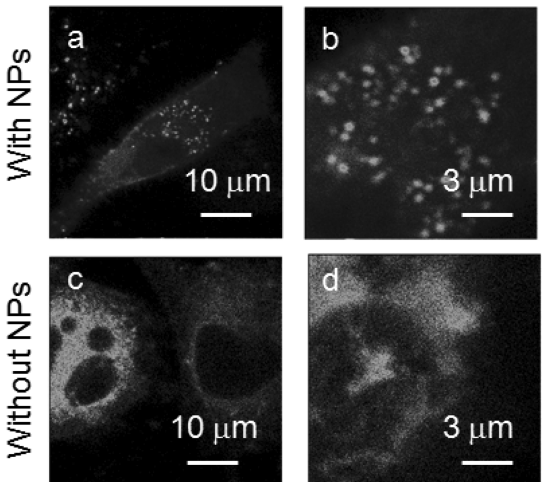

Figure 3. Internalization of AuNPs. (a−d): Fluorescence images of CHO K1 cells when excited with a two-photon excitation microscope. (a and b) cells treated with 20 nm AuNPs, (c and d) control cells. Bright diffraction-limited spots were only seen in the treated samples, in agreement with the presence of nanoparticles.

and forth along the same fiber. Figure 2 shows a typical 3D trajectory when a 20 nm AuNP was moved back and forth along a label-free collagen fiber. Each point in the trajectory represents the position of the AuNP during one tracking cycle (16 ms), while the colors change according to the intensity per dwell time (32 μs). The color scale ranges from 2 MHz (in dark blue) to 150kHz (in dark red), meaning that the local interaction with the fiber determines the level of emission. Regions with different emission properties can be easily identified along the collagen by different colors in the recovered trajectory. For example, the position of the "yellow spots" is highly reproducible when scanning back and forth on the same fiber, meaning that we are mapping the same fiber even if the fiber is moving during the experiment. The temporal *xyz*-coordinates for the center of the orbit and those calculated for the NP position are perfectly superimposed showing that the orbit is always centered with respect to the NP. Another important characteristic of the GENI method that can be demonstrated from Figure 2 is the capability to move the AuNP even if the fiber is slowly moving. Note from Figure 2 that during the experiment, the entire fiber moves near the resolution of the microscope (~400 nm). However, as the GENI method is based on a tracking principle, if the fiber's movement is slower compared with the orbital scanning speed, its overall movement is recorded, but it does not affect the determination of the 3D structure of the fiber since the orbit is always on the fiber.

To illustrate the broad range of biological applications with the GENI method, we moved a AuNP along F-actin filaments inside cells. Chinese hamster ovary (CHO) K1 cells/AuNPs samples were prepared, as described in the Supporting Information. Briefly, 20 nm AuNPs were incubated overnight in the culture medium with CHO K1 cells previously starved in low-glucose media for 2 h at 37 °C. The internalization of NPs by endocytosis has already been reported.[12] Cells uptake NPs from the culture medium, and the resulting endosomes interact with cellular components, such as actin filaments. To visualize F-actin, we labeled the filaments with a fluorescent derivative of phalloidin or by using EGFP-actin for fixed and live cells, respectively. For fixed cells, we selected 488 Alexa-phalloidin as it can be excited with a two-photon excitation microscope. The internalization of the AuNPs was confirmed by fluorescence microscopy, and the identification of the single gold NPs was done exploiting the spectral characterization also described in Supporting Information. Examination of the fluorescence image upon labeling revealed the close proximity of some AuNPs with actin filaments (Figure 3). Control samples without AuNPs were also prepared (Figure 3). First, we performed experiments on fixed cells. We tracked, pushing back and forth, AuNPs near the membrane of CHO K1 fixed cells. The recovered actin topography during the back and forth AuNP's movement shows high reproducibility, indicating that the filaments are almost stationary, which corresponds to the fact that the cells are fixed (Figure 4). To confirm the accuracy of the method, in Figure 4b we remeasure the same actin filament as in Figure 4a using the same AuNP but starting in a different filament's position (indicated by the arrows). A comparison of the recovered trajectories shows that the AuNP follows the same filament, even if the filament is immersed in a filament network. These results strongly support that the GENI method shows the underlying nanometer structure of the F-actin. Finally, to correlate the recovered trajectories with the fiber's topography, we studied the trajectories at the single trajectory level. This is done by analyzing a portion of the total trajectory in a given direction. To distinguish this trajectory from the rest of the trajectories, we highlighted it in a different color in Figure 4c. At the AuNP's single trajectory level, it is possible to observe that the AuNP follows a helicoidally pattern around the actin filament when it is moved by the laser. The period and the amplitude of the helicoidal trajectory are 46 ± 9 and 10 ± 5 nm, in agreement with the structure of actin filaments reported previously.[13,14] This result shows for the first time the capability to obtain nanometer structural information of actin filaments in live cells under physiological conditions using an easy-to-implement optical method. Finally, we performed in vivo experiments to evaluate the performance of the GENI method in a more relevant biological scenario. Figure 5 shows the 3D trajectory of a 20 nm

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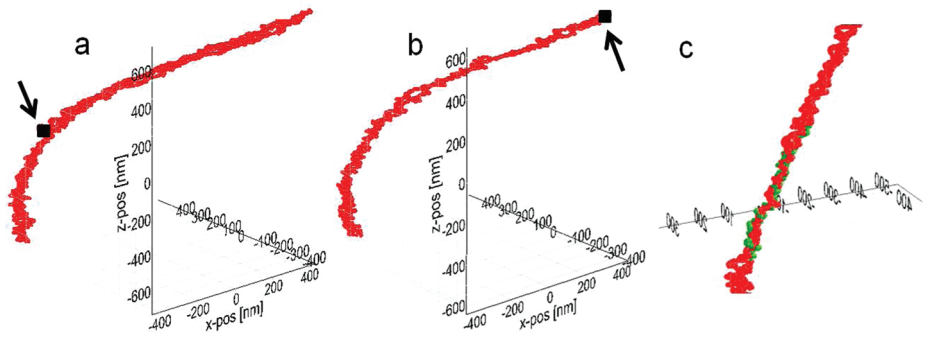

Figure 4. GENI method on fixed cells. (a) 3D trajectory reconstruction of a F-actin filament inside a CHO K1 fixed cell created by moving the AuNP's position back and forth four times. The recovered actin topography during the back and forth AuNP's movement is highly reproducible in agreement with the fact that the cells are fixed. The arrow shows the initial AuNP's position. All the dimensions are in nm. (b) Same measurement as in (a) but with different AuNP's starting position. (c) Actin filament single trajectory. The period of the recovered helicoidally pattern is 46 ± 9 nm.

AuNP when pushed back and forth along an actin filament inside a CHO K1 live cell (Figure 5a) as well as the corresponding fluorescence images (Figure 5b–d). The recovered 3D trajectory shows that the filament is remodeling and moving during the experiment. The fiber's movement is in agreement with the results in collagen fibers (Figure 2).

In conclusion, we describe a method that allows controlled movement of AuNPs along fibers using a two-photon excitation microscope. The GENI method exploits the advantages of particle tracking techniques described previously.[15,16] The principle is similar in concept to methods like fluorescence imaging with one nanometer accuracy (FIONA) that have been successfully used to track the movement of motor proteins along myosin and kinesin filaments.[17−20] However, in addition to the determination of the particle's center of mass (that can be measured with arbitrary precision by collecting enough photons),[21] the GENI method is based on the kicking (follow by the sequentially tracking) of a single AuNP. The implementation of the GENI method allows tracking of single actin filaments inside a live cell even in the presence of many fibers. Control experiments, moving AuNPs along a collagen fiber, were done to demonstrate that the GENI method can move AuNPs (Figure 1b and c) and recover their trajectories even if the fiber changes position during the experiment (Figure 2). Then, by combining the GENI method with conventional fluorescence microscopy, it was possible to move in a controlled manner the AuNP and to observe its final position just after the experiment (Figure 1b and c). Next, we performed experiments of AuNPs moving along F-actin. Analysis of F-actin kinetics in the lamellipodia of cells is a crucial issue, directed toward of a comprehensive understanding of the cell migration process. Figures 4 and 5 show that the AuNP's 3D trajectory can be measured inside cells with high reproducibility, providing detailed information of the topography of filamentous features in living cells with unprecedented detail. In this article we demonstrate a novel optical nanoimaging method that makes use of the high-precision 3D particle tracking of metallic nanoparticles and the photoacoustic effect reported previously to obtain 3D fluorescence images of nanometer-sized, label-free, moving collagen fibers and actin filaments inside living cells. The method not only follows the moving nanoparticle providing the trajectory of the center of mass but also can also promote the nanoparticle's movement in a controlled manner with high spatial and temporal resolutions, thus offering dramatic advantages with respect to conventional particle tracking

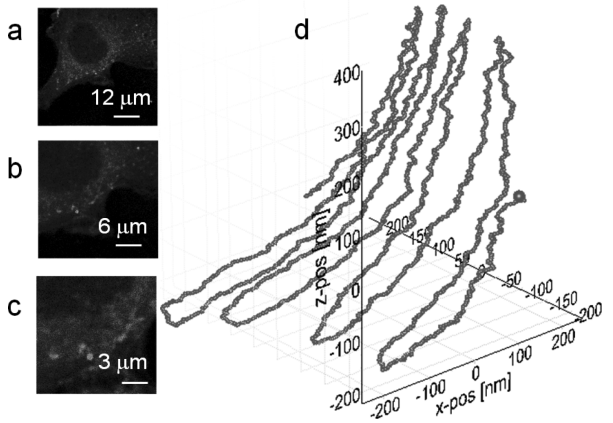

Figure 5. GENI method on live cells. (a−c) Fluorescence images while illuminating with a two-photon excitation microscope. (d) 3D trajectory reconstruction of an F-actin filament inside a CHO K1 live cell created by moving a 20 nm AuNP's position back and forth along the same filament nine times.

methods. Furthermore, a modulation in the emitted signal is visible when the nanoparticles interact with the fibers (Figure 2). This observation is incompatible with a constant emission from the gold and suggests that the NP's emission is modulated by the collagen chromophores on the fiber. We have observed this emission enhancement and showed that it is reproducible when we map the same fiber back and forth. As the use of metallic nanoparticles contribute not only to enhance the signal of the fibers but also to confine the incident electromagnetic field, the detailed study of the emission properties during nanoparticle movement will be the objective of future work. As an example of application of the GENI method, we are currently using this method to explore the dynamics of F-actin in cells during cell migration. Cell migration involves a continuous assembly of F-actin at the cell leading edge, followed by depolymerization in the cell rear.[22] This crucial event in cell movement, however, is poorly understood.

■ ASSOCIATED CONTENT

Supporting Information. Schematic representation of the GENI method (Figure S1), sample preparation and characterization as well as detailed description of the homemade two-photon excitation microscope used for the GENI experiments

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are available. Traditional AuNP 3D tracking experiment can be found in Figure S3 for comparison with the GENI method introduced in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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