Facile method for CLSM imaging unfunctionalized Au nanoparticles through fluorescent channels

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Abstract The microscopic visualization of metal nanoparticles has become a useful tool for the investigation of their applications in cell labeling and the study of their bio-effects. In the current study, we have developed a facile method with confocal laser scanning microscope (CLSM) to observe un-functionalized Au nanoparticles through fluorescent channels. The sharp reflected signal and photostable property of the metal nanoparticles makes the present method very ideal for fluorescent co-localization, real-time imaging, and further quantitative analysis.

Keywords Au nanoparticles · CLSM · Real-time imaging · Instrumentation · Nanomedicine

Introduction

Recently, metal nanoparticles have been widely used to label macromolecular and organelles in biomedical research (Yi et al. 2001; Liu et al. 2003; Tkachenko et al. 2004; De Wall et al. 2006). The bio-effects of these metal nanoparticles have also attracted booming interests (Bhattacharya et al. 2007; Pan et al. 2007). Consequently, the visualization of metal nanoparticles by microscopy has become an invaluable tool. Up to the present, scanning electron microscope (SEM) and transmission electron microscope (TEM) are mostly used imaging methods for fine structure and show successful applications with their individual advantages (Inoue et al. 1982; Tseng et al. 1984; Murata et al. 1985; Geanacopoulos and Gear 1988). However, the sample preparation for SEM and TEM prevents the observation of living cells. Although many works have been contributed for the atomic force microscope (AFM) observation of the living cells in physical solutions (Haberle et al. 1992; Kienberger et al. 2003; Rhode et al. 2004), the inner structure of the cells still could not be revealed.

Confocal laser scanning microscope (CLSM) has been widely performed for fluorescent imaging of cells and tissues, which offers colorized in-focus
images of thick specimens (White et al. 1987; Itoh et al. 1997; Hoffman et al. 2006; Patel and McGhee 2007). As for metal nanoparticles, two ways are always used for the CLSM observation. Some publications have reported the observation of metal nanoparticles through optical field via the surface plasmon resonance (SPR) mechanism (Alschinger et al. 2003), but the advantage of fluorescent co-localization by CLSM cannot be exploited.

As an alternative, researchers also developed methods to functionalize the metal nanoparticles with fluorescent reagents (Shukla et al. 2005; Li et al. 2007) for direct CLSM fluorescent imaging. Unfortunately, fluorescence quenching posed a hurdle for the real-time imaging. The presence of the fluorochrome may also influence the final charge and surface properties of the particles, which would change the intrinsic interactions between these nanoparticles and cells (Delie 1998). Therefore, it is still highly expected to develop a method for the CLSM observation of unfunctionalized metal nanoparticles. Herein, we report an intriguing and facile method with CLSM to image the metal nanoparticles based on their own reflected signals. Au nanoparticles with a diameter of ca. 50 nm were chosen as a prototype to testify the feasibility of this novel method for practical applications (see TEM image of the Au nanoparticles in Supplementary Information Fig. 1).

**Experimental**

DAPI, FITC, and Rhodamine were all ordered from Molecular Probes. DMEM media and fetal bovine serum (FBS) for cell culture were purchased from Gibco. Other reagents were of analytical grade. TEM (H8100, Hitachi) was occupied to observe the Au nanoparticles conventionally, whereas CLSM (TCS SP2, Leica) was used to develop the new method in this manuscript.

The detailed synthetic method of the Au nanoparticles used in this study was reported elsewhere (Frens 1973). Briefly, 2 mL sodium citrate aqueous solution (1.0 wt%) was added to 50 mL chloroauric acid aqueous solution (0.01 wt%) under vigorous stirring. After 12 h, the formed Au nanoparticles were centrifuged at 15,000 rpm at 10 °C for 30 min. These Au nanoparticles were filtered through a 0.22 μm filter and UV-irradiated for 30 min before use in vivo studies. The human hepatocellular carcinoma 7701 cells of passages 35–40 were plated at a density of 2.0 × 10^5 cells/cm² in six-well plates. On days 3 or 4 of culture, the cells formed confluent monolayer. Au nanoparticles were added into the culture medium without FBS at a concentration of 0.17 nM. Cells were then incubated for 6 h and the nanoparticles adhering on the cell surface were removed by washing the cell monolayer three times with ice-cold phosphate-buffered saline. For the CLSM imaging, the cells were stained with DAPI (nuclear dye), FITC, and Rhodamine (cell background), which were excited at 364, 488, and 543 nm, respectively. The corresponding fluorescent images were taken at 430–480, 510–550, and 560–600 nm.

**Results and discussion**

**Reflected signals detected through fluorescent channels**

We found that the signal from the Au nanoparticles could be obtained at the same wavelength with the exciting laser beam (Scheme 1). It is proposed that the beams are easily reflected by metals because of their high reflective extinction coefficient. When the metal nanoparticles were excited with a specific laser beam, the detector could detect the reflected laser signal of the same wavelength. As shown in Fig. 1, when the samples were excited with 633 nm beam, the images of Au particles could be observed when the detector was tuned at 625–637 nm, and no signal was detected out of this narrow range. In addition, we also noticed that there was a linear relationship between the exciting laser power and the reflected light intensity from Au nanoparticles (Fig. 2). Therefore, images of Au nanoparticles with tunable intensity could be precisely controlled by adjusting the laser power during the observation.

**Advantages for fluorescent co-localization, real-time imaging, and quantitative analysis**

Fluorescent co-localization is one of the most useful functions for CLSM. Based on this interesting reflecting phenomenon, we can observe the samples through different fluorescent channels. As shown in Fig. 3, the nuclei dyed with DAPI were observed...
when illuminated with 364 nm beam, and the corresponding fluorescent image was taken at the 430–480 nm channel. Compared with the control image shown in Fig. 3c, the Au nanoparticles are clearly depicted (Fig. 3d), and the corresponding reflected signal was taken in a narrow range around 633 nm (Fig. 3b), which demonstrates excellent discrimination from other fluorescent signals of the labeled organelles. Furthermore, there is a precisely controlled motor equipped on the z axis of CLSM, which allows a fine (≤1 μm) step adjustment and a sharp XY images recording at z dimension. Similarly, a Y-pile of XZ views can also be collected by adjusting the Y position. All these series shown in Fig. 4 could be further constructed by advanced software for 3D imaging and analysis. In addition, there are also some other combinations between the Au nanoparticles and fluorochromes, which make this method very versatile for practical observation of various purposes (see the corresponding spectrum and multi-fluorescent images in the Supplementary Information Figs. 2, 3, and 4).
Fig. 2 Images of Au particles excited at 633 nm with different laser power: a 0 mW, b 2 mW, c 4 mW, and d 8 mW. The linear correlation between laser power and the reflected light intensity is shown in (e) (scale bar: 1 μm)

Fig. 3 Observation of Au nanoparticles in living 7701 cells. a Presents the CLSM manipulation interface, which was performed to observe 7701 cells alone (c) and cells incubated with Au nanoparticles (d). In each panel, the nuclear dyed with DAPI was shown on the left, the Au nanoparticles in the middle, and the overlay on the right (scale bars represent 10 μm). b Displays the corresponding fluorescent spectrum of DAPI and reflected signal of Au nanoparticles in (d) (scale bar: 10 μm)
In addition to the other functions of CLSM, real-time imaging has become highly expected because of its potential in monitoring the kinetics of physical, chemical, and biological processes. Conventional real-time imaging of Au nanoparticles was performed by the conjugation of fluorochrome on the Au nanoparticles.

Unfortunately, fluorescence quenching occurred in most of fluorochrome, such as DAPI, FITC, and Rhodamine, which posed a hurdle for non-instant scans. On the contrary, little quenching was detected on Au nanoparticles using the present method (Fig. 5), since the light was sourced from the reflection. Such a photostability

**Fig. 4** XY–Z (a) and XZ–Y (b) series of living 7701 cells incubated with Au nanoparticles (scale bar: 10 μm)

**Fig. 5** Comparative photostability of DAPI and Au nanoparticles. a The original nuclei (DAPI) with Au nanoparticles. b Quantitative quenching assays of DAPI and Au nanoparticles. c and d Present the corresponding images of nuclei (DAPI) and Au nanoparticles during the assays, respectively (scale bar: 10 μm)
enables the real-time and consecutive data collection for a long time range, thus providing the critical insight into the fundamental nature of the interaction between the metal nanoparticles and cells.

Data acquired with CLSM/Au nanoparticles method can be easily analyzed in a quantitative manner by digital image process (see the details in the Supplementary Information Fig. 5), while images taken by SEM, TEM, and AFM cannot be directly applied for quantitative analysis of the same purpose. The highest horizontal resolution of most CLSM available in the market is about 150–200 nm (Prasad et al. 2007), which determines the sensitivity of this imaging method for the quantitative analysis.

Conclusion and future prospect

In summary, a facile method has been developed to visualize unfunctionalized Au nanoparticles by CLSM through fluorescent channels. The photostable property and sharp reflected signal of the Au nanoparticles make the present method very ideal for fluorescent co-localization, real-time imaging, and further quantitative analysis. Recently, a new product STED CLSM (Leica) has overcome the limits imposed by diffraction, and a higher resolution (below 100 nm) has been achieved which would also make the present method very promising to trace the fate of a single Au nanoparticle in the living cell. In addition to Au nanoparticles, we also noticed that some other metal nanoparticles, such as Ni and Pd nanoparticles, could also be observed by CLSM following the same procedures as reported above (see the details in the Supplementary Information Fig. 6). We propose that this report and the procedures described herein will help explore the applications of CLSM in new research fields and develop CLSM as a powerful tool for imaging and analysis of metal nanoparticles in the study of biological and medical sciences.

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References


