

# Single-molecule imaging in live cell using gold nanoparticles

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## Abstract

Optimal single particle tracking experiments in live cells requires small and photostable probes, which do not modify the behavior of the molecule of interest. Current fluorescence-based microscopy of single molecules and nanoparticles is often limited by bleaching and blinking or by the probe size. As an alternative, we present in this chapter the synthesis of a small and highly specific gold nanoprobe whose detection is based on its absorption properties. We first present a protocol to synthesize 5-nm-diameter gold nanoparticles and functionalize them with a nanobody, a single-domain antibody from camelid, targeting the widespread green fluorescent protein (GFP)-tagged proteins with a high affinity. Then we describe how to detect and track these individual gold nanoparticles in live cell using photothermal imaging microscopy. The combination of a probe with small size, perfect photostability, high specificity, and versatility through the vast existing library of GFP-proteins, with a highly sensitive detection technique enables long-term tracking of proteins with minimal hindrance in confined and crowded environments such as intracellular space.

## INTRODUCTION AND RATIONALE

Over the last decades, single-molecule imaging has been widely used to describe the organization and dynamics of biomolecules at the nanometer scale and to unravel complex cellular processes in live cells (see also Ananthanarayanan and Tolic [Chapter 1 of this volume] and Yamashiro et al. [Chapter 4 of this volume]). Recent developments in single-molecule imaging involve improvements on two complementary aspects: the microscopy techniques allowing probe detection with always better spatial and temporal resolutions, and the probe synthesis allowing better biocompatibility, brightness (for precise detection), optical stability (for longer detection), specificity, monovalency together with a size as small as possible in order not to perturb the molecular function.

Because of its high sensitivity and noninvasiveness, the most frequent techniques used to detect single molecules in biological environments are based on fluorescence microscopy (Deschout et al., 2014). They benefit from a large toolbox of labeling strategies with fluorescent proteins (Shcherbakova & Verkhusha, 2014), organic dyes (coupled with SNAP-tag, fluorescent antibodies etc. (Chen, Cornish, & Min, 2013; Correa, 2014)), or quantum dots (Pinaud, Clarke, Sittner, & Dahan, 2010). Advancements in fluorophore chemistry allowed the development of super-resolution microscopy. However, single-fluorophore imaging also suffers from a major drawback coming from photobleaching, which limits the observation time to typically a few seconds (at video rate) and prevent long-term tracking (Cognet, Leduc, & Lounis, 2014). On the contrary, quantum dots have longer observation

time but at the expense of bigger size ( $\sim 20$  nm with functionalization), blinking, and lower biocompatibility, which impact significantly the dynamical properties that are measured (Groc et al., 2007).

Alternative methods to fluorescence microscopy involve labeling with nonfluorescent nanoparticles made of latex, silica, polystyrene, or metal. The detection of such nanoparticles usually relies on Rayleigh scattering using standard microscopy techniques, but it is limited to particles bigger than 40 nm (Cognet et al., 2014). Such large diameters are not suitable to track biomolecules in confined cellular environments such as adhesion sites and synapses. In contrast, detection techniques that depend on the probe absorption properties do not suffer from such size limitation, nor photobleaching or blinking, making them attractive for robust single-molecule detection. Indeed, gold nanoparticles have a relatively large absorption cross-section ( $6 \times 10^{-14}$  cm<sup>2</sup> for a 5-nm-diameter gold nanoparticle) when they are excited near their plasmon resonance, and exhibit a fast electron–phonon relaxation time in the picosecond range, which makes them very efficient light absorbers (Berciaud, Lasne, Blab, Cogne, & Lounis, 2006). Photothermal heterodyne imaging (PHI) technique (Berciaud, Cogne, Blab, & Lounis, 2004) has proven to be a valuable detection method because it allows the imaging of 5-nm gold nanoparticles with potentially unlimited observation times and stable optical signals, which are insensitive to the scattering environment of the cell (Lasne et al., 2006). However, to keep the probe size as small as possible, it is important that the ligand used to bind the particle to the targeted biomolecule also remains small. Lasne et al. (2006) used gold nanoparticles coated with secondary antibodies coupled to primary antibodies targeting AMPA receptors ( $\sim 12$  nm size each). Apart from the size issue, the use of antibodies is also not ideal, because they often have a limited affinity to the protein of interest and are always divalent.

Here we describe a protocol to synthesize 5-nm gold nanoparticles and functionalize them with nanobodies, a small fragment of camelid antibody ( $2 \times 4$  nm), which recognizes green fluorescent proteins (GFPs) ( $3 \times 4$  nm) with a very high affinity ( $K_d \sim 0.23$  nM) (Rothbauer et al., 2006). We explain how to label proteins at the cell surface and how to internalize the nanoparticles to target intracellular proteins. We also describe in details the PHI setup necessary to image and track such nanoparticles. The present protocol allows the production of purified and monovalent probes, which have been recently used to detect and track all sorts of GFP-tagged proteins in confined structures and inside living cells using PHI (Leduc et al., 2013).

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## 1. GOLD NANOPARTICLE SYNTHESIS AND FUNCTIONALIZATION

### 1.1 MATERIALS

#### 1.1.1 List of chemicals required for the nanoparticle synthesis and functionalization

- HAuCl<sub>4</sub> 3H<sub>2</sub>O (Very hygroscopic, use a Teflon spatula during weighing, store tightly capped, and store in refrigerator) (Aldrich, Cat. No.: 520918-1G)

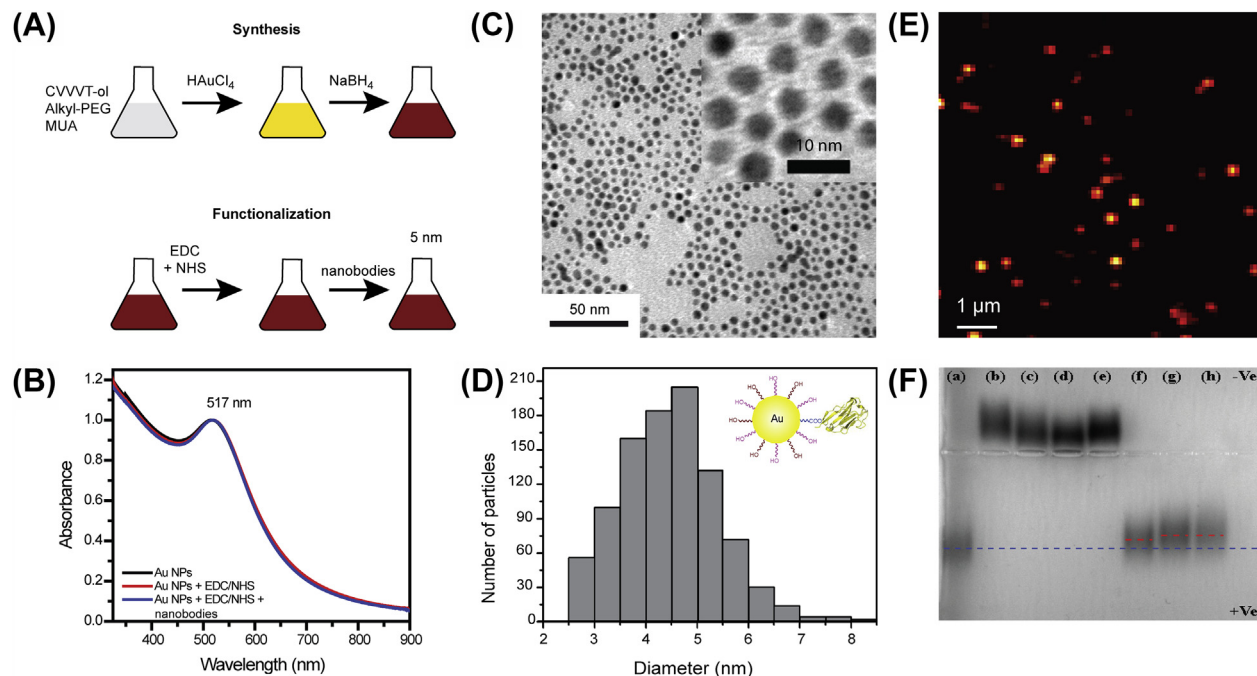
- Methanol (Aldrich, Cat. No.:320390-1L)
- Acetic acid (Aldrich, Cat. No.: 695092-100ML)
- CVVVT-ol peptide (Store at  $-20\text{ }^{\circ}\text{C}$ ) (Activotec, UK, Cat. NO.: Custom Peptide)
- PEGylated alkanethiol, HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>4</sub>-OH (alkyl PEG) (store tightly capped at  $-20\text{ }^{\circ}\text{C}$ ) (Prochimia, Cat. No.: TH 003-02)
- 11-Mercaptoundecanoic acid (MUA) (store tightly capped at  $-20\text{ }^{\circ}\text{C}$ ) (Aldrich, Cat. NO.: 450561-5G)
- Sodium borohydride (very hygroscopic, prepare the solution just before its use) (Fluka, Cat. No.: 71321-25G)
- Mili-Q water (resistivity = 18.2 mΩ)
- Tween20 (Sigma, Cat. No.:P7949-100ML)
- Phosphate buffer saline (PBS) (Sigma, Cat. NO.: P5493-1L)
- 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (hygroscopic and corrosive, store at  $-20\text{ }^{\circ}\text{C}$ ) (Fluka, Cat. No.: 03450-5G)
- N-Hydroxysuccinimide (NHS) (Aldrich, Cat. No.: 130672-5G)
- Nanobodies (Store at  $-20\text{ }^{\circ}\text{C}$ )
- Glycine (Sigma, Cat. No.: G7126-100G)
- Membrane filtration unit (Amicon Ultra centrifugal filter units Ultra-4, MWCO 30 kDa) (Sigma, Cat. No.: Z648035-24EA & Z677892-24EA)

## 1.2 NANOPARTICLE SYNTHESIS

The 5-nm gold nanoparticles are prepared by a single-step borohydride reduction of gold salt in the presence of a mixture of ligands (CVVVT-ol, short alkyl PEG, and 11-MUA) using a protocol modified from (Duchesne, Gentili, Comes-Franchini, & Fernig, 2008; Zheng & Huang, 2004) (Figure 1(A)). Equimolar amounts of the peptide and alkyl PEG are used, whereas various amounts of the functional ligands MUA can be used from 20% of the total ligand concentration to 0.4%.

For 20% MUA:

- 4 mg (0.008 mmol) of CVVVT-ol, 3.04 mg (0.008 mmol) of short alkyl PEG, and 0.87 mg (0.004 mmol) of MUA are added to a mixture of solvents containing methanol (3.0 mL) and acetic acid (0.5 mL) and dissolved by stirring.
- Add 0.05 mmol (19.7 mg) HAuCl<sub>4</sub> 3H<sub>2</sub>O to the above partially dissolved ligand mixture. Keep stirring the solution for at least 5 min; the solution will become transparent and yellow.
- Prepare a sodium borohydride solution by dissolving 30 mg of NaBH<sub>4</sub> in 1.5 mL of ice-cold milli-Q water.
- Add dropwise the NaBH<sub>4</sub> solution into the gold solution with rapid stirring. The color of HAuCl<sub>4</sub> will change from yellow to brown upon addition of NaBH<sub>4</sub> solution.
- Continue the rapid stirring for 4 h at room temperature.
- Add 25 μL of 1% Tween20 and then keep overnight with slow stirring. The addition of Tween20 helps in minimizing the sticking of the nanoparticles to the surface of the glass container as well as increases the dispersibility in the medium.

**FIGURE 1**

Synthesis and characterization of 5-nm-diameter gold nanoparticles coated with nanobodies. (A) Sketch of the different steps of synthesis and functionalization. (B) UV-visible absorption spectra of the gold nanoparticles after synthesis (black), functionalization (red), and coupling with nanobodies (blue). (C) Transmission electron microscopy image (TEM) of the gold nanoparticles after nanobodies conjugation. (D) Histogram showing the size distribution extracted from the TEM images. (E) Photothermal image of gold nanoparticles after nanobodies conjugation trapped in a PVA matrix. (F) Agarose gel electrophoresis of gold nanoparticles with various functionality: (a) 20% MUA, no nanobodies; (b) 1% MUA, no nanobodies; (c) 0.4% MUA, no nanobodies; (d) 0.4% MUA, equimolar nanobodies; (e) 1% MUA, equimolar nanobodies; (f) 20% MUA, equimolar nanobodies; (g) 20% MUA, twofold excess nanobodies; and (h) 20% MUA, 10-fold excess nanobodies.

- Filter the particle solution by centrifugation through a 30-KDa molecular weight cutoff membrane filter and wash with methanol containing 0.005% Tween20 (four times) and with PBS containing 0.005% Tween20 (two times) (5 min at 5000 rpm at 20 °C).
- Dissolve the solution in a minimum amount of PBS containing 0.005% Tween20. Further dilution with PBS containing 0.005% Tween20 will be carried out to maintain the gold nanoparticle OD at  $\sim 1$  for further nanobodies conjugation (concentration  $\sim 0.1 \mu\text{M}$ ) (Figure 1(B)). Very slight aggregation (caused by the particles that are not well coated by the ligands) may appear in the diluted solution. Such aggregates are separated and removed by filtering the solution through a 0.22- $\mu\text{m}$  millipore filter before nanobodies conjugation.

$$[\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}]:[\text{Ligand}] = 0.05:0.02 \text{ (mmol)}$$

$$\text{Ligand} = \text{CVVVT-ol} + \text{alkyl PEG} + \text{MUA}$$

$$[\text{MUA}]:[\text{CVVVT-ol}]:[\text{alkyl PEG}] = 0.004:0.008:0.008 \text{ (mmol)} = 20:40:40 \text{ (\%)}$$

Note: Calculate the ratio between the different ligands as desired to maintain the same ratio of  $[\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}]:[\text{Ligand}] = 5:2$  to get  $\sim 5\text{-nm}$ -diameter gold nanoparticles.

### 1.3 NANOPARTICLE FUNCTIONALIZATION WITH NANOBODIES

- Prepare a solution of nanobodies, which can be kept for a few months at  $-20 \text{ }^\circ\text{C}$  in a PBS–glycerol (1:1) mixture.

A llama cDNA encoding GFP nanobody has been synthesized and optimized for *Escherichia coli* expression. The resulting cDNA has been cloned into pET28a (Novagen) that fuses a C-terminal 6-His-tag to the GFP nanobody sequence. The fusion protein is purified using classical  $\text{Ni}^{2+}$  sepharose chromatography.

- Add 50  $\mu\text{L}$  of freshly prepared 500 mM freshly prepared EDC and 500 mM NHS to 0.5 ml of nanoparticle solution (concentration  $\sim 0.1 \mu\text{M}$ ) in PBS buffer solution.

EDC and NHS are not stable in solution.

- Mix thoroughly with a vortex and let incubate for 15 min at room temperature.
- Purify the nanoparticle solution by centrifugal filtration (5 min at 5000 rpm at 20 °C). Wash once with PBS and then redisperse in 0.5 mL of PBS.
- Add the required amount of nanobodies directly to the nanoparticle solution and mix using a vortex. We used typically 1  $\mu\text{L}$  of nanobodies at 0.8 mg/mL for 0.5 mL nanoparticle solution (final concentration  $\sim 0.1 \mu\text{M}$ ).
- Let incubate for 4 h at 4 °C.
- Add 20  $\mu\text{L}$  of 0.01 M glycine to quench the unreacted active sites.
- After another 30 min, purify the nanoparticles by centrifugal filtration (30-kDa filter, 5 min at 5000 rpm at 4 °C) and wash three times with PBS by repeating centrifugal filtrations. Finally, redisperse nanoparticles in 100  $\mu\text{L}$  of PBS.

- Facultative step: Nanobodies-coated nanoparticles can be separated from the nonfunctionalized nanoparticles by chromatography using cobalt resin (Qia-gen). This step is useful only for intracellular labeling.
- Store the nanobodies-coated nanoparticles at  $-20\text{ }^{\circ}\text{C}$  and use them in the next 3 months.

There are two possible approaches to obtain monovalent nanoparticles. The first method consists in adding a low amount of nanobodies into an excess of synthesized nanoparticles bearing an excess of linkers MUA (typically 20% of the coating). The second method consists on decreasing as much as possible the amount of linkers MUA compared to the blocking ligand (CVVVT-ol/PEG) in order to have at most one linker per particle (typically 0.4% of the coating). Nanoparticles are then mixed with an excess of nanobodies to optimize the coupling. Interestingly, the first method bears the advantage not to require a large quantity of nanobodies compared to the second one. Moreover, it is possible to separate the functionalized nanoparticles from the nonfunctionalized ones using the His-tag of the nanobodies and a cobalt resin. This last step is facultative and can be useful for intracellular labeling of GFP-proteins (cf. II.d).

## 1.4 SAMPLE CHARACTERIZATION

### 1.4.1 Absorption spectra

After synthesis and functionalization, the concentration of gold nanoparticles is calculated from the absorbance measurements using a spectrophotometer (Figure 1(B)). Gold nanoparticles having diameter of 5 nm display a moderately sharp absorption band at 517 nm, the plasmon resonance, which is characteristic for such sizes. Activation of the surface carboxylic group with EDC-NHS and subsequent nanobodies coupling does not alter the position and nature of the absorption band, indicating that the size/dispersibility of the gold nanoparticles remains unaltered even after nanobodies conjugation. The nanoparticles produced are therefore stable against further chemical treatment and this is the signature of an appropriate functionalization with a mixed ligand system.

### 1.4.2 Transmission electron microscope

Ten microliters of the nanoparticle sample was put onto a carbon-coated 400-mesh copper grid and let dry. Transmission electron microscopy (TEM) images were collected at room temperature on a HITACHI H7650 TEM using an accelerating voltage of 100 kV. The nanoparticle diameter is measured from TEM images (Figure 1(C)) and the size distribution is extracted using Image J (Figure 1(D)). No significant difference in diameter is observed between functionalized and non-functionalized nanoparticles with an average diameter of  $4.5 \pm 1\text{ nm}$  (SD).

### 1.4.3 Agarose gel electrophoresis

To provide further evidence of the conjugation between the nanobodies and the gold nanoparticles, a titration is followed by agarose gel electrophoresis (Dif et al., 2009).

Figure 1(F) shows the mobility of three different sets of nanoparticles coated with 20% (Figure 1(F) a, f, g, h), 1% (Figure 1(F) b, e), and 0.4% (Figure 1(F) c, d) of the linker ligand MUA. The nanoparticles with 20% MUA show greater mobility and move faster toward the positive electrode, indicating a net negative charge on the nanoparticles. The nanoparticles with a lower amount of MUA (1% and 0.4%) move toward the negative electrode but with slower velocities. This may be due to a competition between positive charge of the CVVVT peptide and negative charge of MUA and thus the net charge on these particles is slightly positive. Nanoparticles mobility is significantly modified after nanobodies conjugation, which confirms that the conjugation took place.

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## 2. PHOTOTHERMAL IMAGING

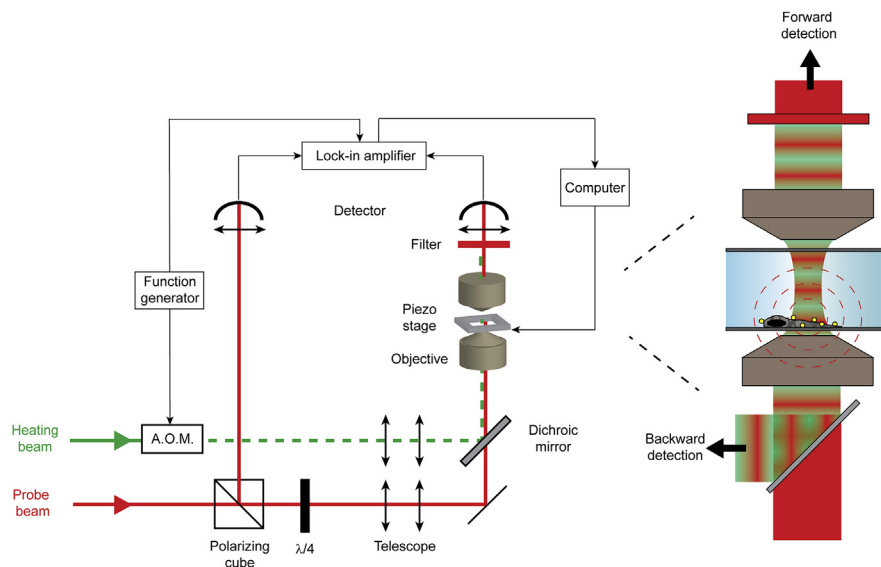
### 2.1 MATERIALS

- Optical table, lense, quarter wave plate, polarizing cube
- Commercial inverted microscope with a Xe or Hg lamp for fluorescent measurement, a CCD camera, and a side port used for the photothermal imaging
- x60, NA = 1.49 oil objective
- x60, NA = 1.0 water objective
- Frequency doubled Nd:YAG laser, 532 nm
- Helium–Neon laser, 20 mW
- Acousto-optic modulator (AA Optoelectronic)
- Function generator
- Piezo-scanner stage (Physik Instrumente)
- Photodiode (Newport, ref: 2051-FS)
- Lock-in amplifier (Signal recovery)
- Acquisition card (NIDAQ, National Instruments)
- Computer
- LABVIEW, MATLAB softwares

### 2.2 PRINCIPLE

PHI method has first been described in 2004 (Berciaud et al., 2004). It combines a time-modulated heating beam and a nonresonant probe beam that overlap on the sample (Figure 2). The probe beam produces a frequency-shifted scattered field as it interacts with the time-modulated variations of the refractive index around the absorbing nanoparticle. The scattered field is then detected by a photodiode through its interference with the probe field, which acts like a local oscillator. The photothermal signal is extracted from the beat note at the modulation frequency by lock-in detection. The theoretical and detailed experimental characterizations are presented in Berciaud et al. (2006). Further applications to cell biology can be found in Duchesne et al. (2012), Lasne et al. (2006), Leduc et al. (2011), Leduc et al. (2013).



**FIGURE 2**

Sketch of the photothermal heterodyne imaging setup with backward and forward configurations.

### 2.3 EXPERIMENTAL SETUP

The PHI experimental setup is described in [Figure 2](#); it has been built on a commercial epifluorescence inverted microscope to facilitate biological applications. Any researcher with an optics background and programming skills should be able to upgrade his or her microscope with a PHI setup, as long as the commercial microscope has a second side port (in addition to the one used for the camera) used to let the heating and probe lasers beams through. The heating beam (532 nm, close to plasmon resonance of the nanoparticles) and probe beam (632.8 nm) are overlaid and focused on the sample using a high-numerical-aperture objective (60 $\times$ , NA = 1.49). The heating beam intensity is modulated at a frequency of 700 kHz by an acousto-optic modulator, which is controlled by a function generator. The interfering probe-transmitted and forward-scattered fields can be collected either in the forward or in the backward direction ([Berciaud et al., 2006](#)), the backward configuration being more suited to detect nanoparticles close to the bottom glass coverslip. A combination of a polarizing cube and a quarter wave plate is used in the backward configuration, whereas a second objective (water 60 $\times$ , NA = 1) and a filter are used in the forward configuration. In both configurations, the signal is focused on a fast low-noise photodiode, which has a very low dark current. Photothermal images are obtained by sample scanning using a piezo-scanner stage controlled by a LABVIEW routine with 5 ms integration time per pixel. Fluorescent images of the samples are collected on a CDD camera and overlaid with the photothermal images using a MATLAB routine.

## 2.4 RESOLUTION AND SENSITIVITY

### 2.4.1 Resolution

The transverse resolution is given by the transverse dimensions of both laser beams focalized in the sample plane and the temperature profile around the nanoparticles (Berciaud et al., 2006). Experimentally, it is determined by imaging 5-nm gold nanoparticles immobilized in a polyvinyl alcohol matrix ( $\sim 1\%$  mass), by spin coating on clean glass coverslips. The transverse intensity profile of PHI signal from a single nanoparticle displays a full-width-at-half-maximum of 250 nm determined by a Gaussian fit (Figure 3(A) and (B)), and mainly corresponds to the product of the two beams profiles. This width measurement will be used later for single-particle tracking experiments.

### 2.4.2 Sensitivity

If the probe beam intensity is high enough ( $P_i > 1$  mW), the detection is shot-noise limited (Berciaud et al., 2006) and the sensitivity is high enough for the detection of individual gold clusters as small as 1.4 nm in diameter (Berciaud et al., 2004). The photothermal signal is directly proportional to both heating and probe beam intensities. However, it is necessary to limit the heating around the nanoparticles in order to be compatible with live cell measurements. We estimated that an intensity of  $2.5$  MW  $\text{cm}^2$  for the heating beam leads to a local temperature rise of 10 K at the surface of the 5-nm nanoparticles as described in Berciaud et al. (2006). Because the temperature rise decreases as the inverse of the distance from the nanoparticle center, it is only of 2.5 K at 10 nm from the nanoparticle. Such heating intensity together with a  $9$ -MW. $\text{cm}^2$  intensity for the probe beam is sufficient to detect 5-nm gold nanoparticles in an aqueous environment with a signal-to-noise ratio of  $\sim 30$  to 50. Of note, if the synthesized nanoparticles are smaller than 3 nm, the absorption spectra will be larger around the plasmon resonance and so the particles will also absorb the probe beam (at 633 nm). This will provoke extra heating around the nanoparticles and should be avoided. In that case, it is recommended to use a probe beam at 1064 nm as described in Leduc et al. (2011), but single nanoparticles will also be much more difficult to detect due to their small size.

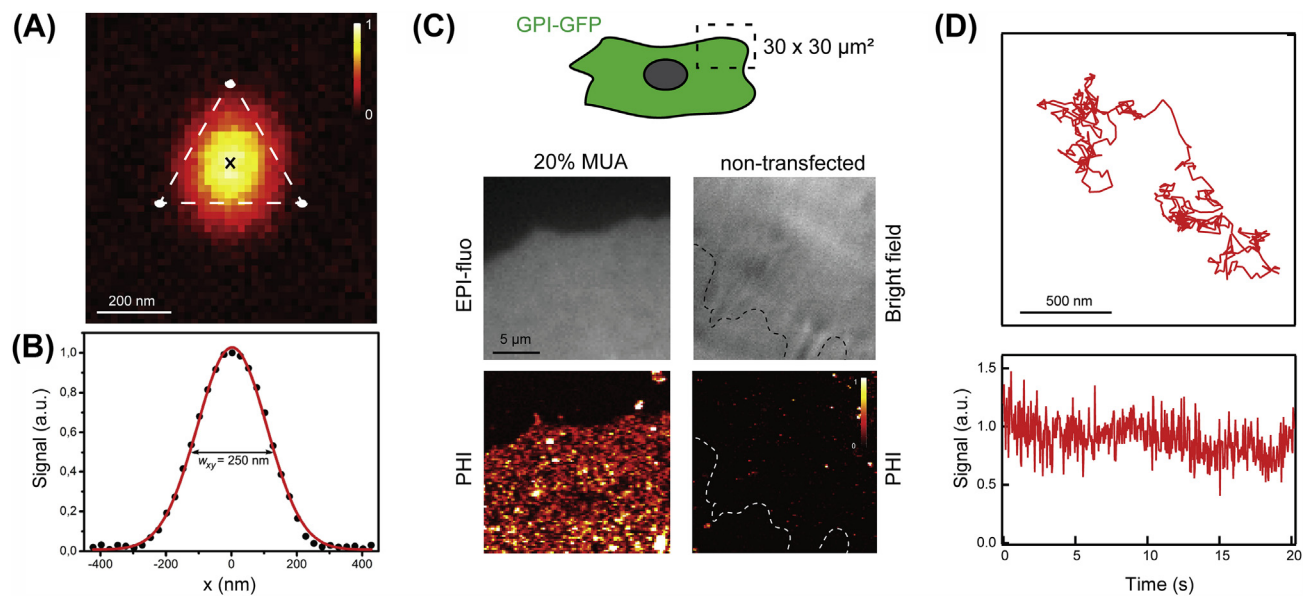
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## 3. LIVE CELL IMAGING

### 3.1 CELL SURFACE LABELING

The binding specificity of the nanobodies-coated nanoparticle is tested by measuring the attachment on the cell surface of COS-7 cells expressing a glycosylphosphatidylinositol (GPI)-anchored proteins with an outer membrane GFP tag (Figure 3(C)). The protocol for cell surface labeling is as follows:

- Plate cells on 18-mm clean glass coverslips in a 12-well plate ( $\sim 30,000$  cells/coverslip). Cells are cultivated in DMEM with 10% fetal bovine serum and 1% penicillin streptomycin.

**FIGURE 3**

Imaging and tracking of single particle in live cells. (A) High-resolution image of a single gold nanoparticle overlaid with the triangle used for single-particle tracking and (B) corresponding PHI intensity profile fitted by a Gaussian. (C) Left: Epifluorescence and corresponding PHI images of COS-7 cells expressing GPI-anchored proteins tagged with an extracellular GFP and labeled nanobodies-coated nanoparticles (20% MUA). Right: control corresponding nontransfected cell. (D) Trajectory of a single nanoparticle attached to a GPI-GFP expressed in a COS-7 cell and corresponding photothermal signal.

- Transfect cells 48 h before imaging. We use FuGENE<sup>®</sup> 6 Transfection Reagent: mix 0.5  $\mu\text{L}$  of DNA at 1  $\mu\text{g}/\mu\text{L}$ , 1  $\mu\text{L}$  FuGENE<sup>®</sup> 6, and 20  $\mu\text{L}$  PBS per well, wait for 5 min, and add dropwise on the cells.

On the day of imaging:

- Mix 10  $\mu\text{L}$  of nanobodies-coated nanoparticles (initial concentration of  $\sim 0.1 \mu\text{M}$ ), 2  $\mu\text{L}$  of a 10 mg/mL solution of casein (Sigma) in PBS, and 88  $\mu\text{L}$  PBS (10 $\times$  dilution).
- Make a droplet on a piece of parafilm.
- Place the coverslip covered by the transfected cells on top of the droplet and let incubate for 10 min at room temperature.
- Wash three times with PBS and mount the coverslip on a sample holder. We used as observation medium a serum-free Ringer's solution.
- Photothermal imaging can be performed 1 h after labeling.

A 10-fold dilution of the nanoparticle sample allows a dense labeling of the cell surface (Figure 3(C)), but is not adapted for single-particle tracking where much lower densities are necessary. Dense labeling of cell surface is useful to compare transfected and nontransfected cells. The absence of particles at the surface of non-transfected cells is a clear proof of the specificity of the nanobodies-coated nanoparticle sample.

Because photothermal imaging is a very sensitive technique, it is able to detect light-absorbing organelles such as mitochondria (Lasne et al., 2007; Leduc et al., 2011). It is important to verify the absence of mitochondria in the region of interest or bleach them by a fast localized scanning (Leduc et al., 2011).

### 3.2 2D SINGLE-PARTICLE TRACKING

Because the required acquisition time to record a  $10 \times 10 \mu\text{m}^2$  PHI image is about 1 min, it is too slow to follow the motion of fast moving single biomolecules. To overcome this limitation, a dedicated strategy has been developed to track single nanoparticle at video rate using a triangulation procedure (Lasne et al., 2006). This method relies on the precise knowledge of the photothermal intensity profile expected for a single nanoparticle, which can be well approximated by a 2D Gaussian with a constant width (Figure 3(A) and (B)). Once a particle is detected, only three measurements around its central position are necessary to determine the precise location in space and the peak intensity signal. Such measurements are performed at the corners of an equilateral triangle with a radius of 1.5 times the width of the Gaussian (Figure 3(A)).

In practice, the single-particle tracking is performed using a dedicated routine written in LABVIEW, which controls the piezo-scanner stage and works as follows:

- A region of interest is chosen in which photothermal signal is measured at random positions.

- When the measured signal is higher than a predefined threshold (typically half of the peak value for a single particle), three data points are taken around this initial position at the corners of an equilateral triangle.
- The position and peak signal are calculated in real time using the 2D Gaussian shape determined previously.
- If the calculated peak signal is still higher than the threshold, the triangle is recentered on the new calculated position for the next three measurements. The procedure is repeated iteratively until the peak signal is below the threshold. The trajectory of a single particle is recorded within about 40 ms per point (Figure 3(D)). Knowing the intensity signal that is expected for a single 5-nm gold particle with the corresponding acquisition parameters, we can estimate the size of the tracked object from its signal intensity and therefore the number of nanoparticles involved. Indeed, the photothermal signal increases with the third power of the nanoparticle radius (Berciaud et al., 2006).

The maximum displacement that can be recorded with this triangulation procedure is about the size of the triangle, namely 180 nm. This corresponds to a maximum diffusion coefficient of  $\sim 0.2 \mu\text{m}^2/\text{s}$  for 5-nm nanoparticles. This maximum value is mainly limited by the integration time necessary to detect the nanoparticles (5 ms per position) and could be improved by using bigger particles, which provide more photothermal signal and require smaller integration time to be detected. On the contrary, the minimal diffusion coefficient is determined by the pointing accuracy and corresponds to  $\sim 0.001 \mu\text{m}^2/\text{s}$ . We measured the pointing accuracy to be  $\sim 11$  nm with a signal-to-noise ratio of 50 and fixed nanoparticles.

Note that there is an alternative technique to measure diffusion coefficients without tracking single particles called photothermal absorption correlation spectroscopy, analogous to fluorescence correlation spectroscopy (Octeau et al., 2009; Paulo et al., 2009; Radunz et al., 2009).

The sample preparation for single-particle tracking is similar to Section 3.1 except that a higher dilution of the nanobodies-coated nanoparticles is used (typically 100-fold instead of 10).

### 3.3 PARTICLE INTERNALIZATION

Due to its high specificity, nanobodies-coated nanoparticles represent a good tool for targeting and tracking intracellular proteins in living cells (Leduc et al., 2013). Due to the limitations of the tracking method (for 5-nm nanoparticles), proteins diffusing slower than  $0.2 \mu\text{m}^2/\text{s}$  in 2D are more suitable for tracking than fast diffusive cytosolic proteins. As described in Leduc et al. (2013), proteins involved in directed motion (speeds up to  $1 \mu\text{m}/\text{s}$ ) or involved in confined structures, such as focal adhesions, would also be appropriate targets.

To perform internalization experiments, it is more effective to use a sample of concentrated and purified nanoparticles ( $\sim 8 \mu\text{M}$  final). We used nanoparticles coated with 20% MUA, which are negatively charged and can therefore be internalized by electroporation.

- COS-7 cells ( $\sim 1$  million) are washed in PBS and detached from their flask with trypsin-EDTA.
- After 5 min centrifugation at 500 g, remove the supernatant and resuspend the pellet in 90  $\mu\text{L}$  of nucleofector<sup>®</sup> solution (Lonza).
- Add 5  $\mu\text{g}$  of the DNA corresponding to the GFP-tagged protein of interest and 15  $\mu\text{L}$  of 8  $\mu\text{M}$  nanobodies-coated nanoparticles.
- Select the appropriate Nucleofector<sup>®</sup> Program W-001 and electroporate the mix.
- Resuspend in 10 mL of preheat culture medium.
- Centrifuge 5 min at 500 g to remove the excess of nanoparticles
- Resuspend in 10 mL of preheat culture medium.
- Plate on 18-mm clean glass coverslips.
- Perform PHI and tracking 12–48 h after plating.

## CONCLUSION

Photothermal imaging is a far-field microscopy technique, which allows the detection of gold nanoparticles with the sensitivity of electron microscopy but with standard optical resolution. Because of its noninvasiveness and its efficiency in diffusive environments, photothermal imaging is also well suited for biological applications when it is associated to appropriate probes. Here we report the synthesis of 5-nm gold nanoparticles coated with nanobodies, which can target GFP-tagged proteins with high affinity and specificity. Such probes are ideal for single-particle tracking in live cells due to their small size and high photostability, and therefore to record the full history of proteins even in highly confined regions such as focal adhesions or synapses. Moreover, these gold nanoparticles can also be perfect probes for imaging with electron microscopy making the photothermal imaging a key technique for correlative microscopy.

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