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**Author:** Carozza, S.

**Title:** Two-photon luminescence of gold nanorods: applications to single-particle tracking and spectroscopy

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SUMMARY

Biomolecular processes occurring inside cells are the basis of life for all organisms, and regulate growth, diseases and death. The main protagonists of these processes are proteins and nucleic acids. When advances in microscopy techniques made it possible to follow single biomolecules during cellular processes, many new insights in life were gained that were unimaginable with ensemble techniques. To perform single-molecule microscopy, biomolecules such as proteins must be functionalized with a suitable optical label. Metal nanoparticles were proposed as promising labels, due to their brightness and photostability as compared to traditionally used fluorescent proteins and dyes. Among nanoparticles, gold nanorods are often preferred, because they can be easily functionalized and they can be excited with low energy photons. In addition, the spectral response of gold nanorods depends on the dielectric constant of their immediate surroundings. This property makes them useful as sensors: measuring the spectrum of gold nanorods can reveal interactions with biomolecules.

In this thesis, we explored the use of gold nanorods as labels for single-particle tracking in live cells and tested the acquisition of excitation spectra of single gold nanorods for sensing applications. We took advantage of two-photon excitation of gold nanorods: non-linear excitation provides a better image contrast and therefore a higher 3-dimensional accuracy, as well as the possibility to use low-energy light, with consequently lower photodamage of cells. Moreover, two-photon excitation spectra are narrower than one-photon spectra, and they therefore provide a more sensitive spectral response. We used a multifocal scanning microscope to perform 3D imaging inside cells with nanometer positional accuracy.

When performing single-particle tracking, mean squared displacement analysis is the most common method to obtain mobility infor-
mation from trajectories of tracked particles, such as the diffusion coefficient. The precision of the diffusion coefficient sets a limit to discriminate changes in mobility, caused by biological events, from the statistical variation inherent to diffusion. This issue is therefore of particular importance in experiments aiming to quantify dynamic processes. In Chapter 2 we presented simulations and 3D tracking experiments in vitro, using gold nanorods freely diffusing in glycerol solution, to establish the best analysis parameters to extract the diffusion coefficient. We applied this knowledge to the detection of a temporary change in diffusion, as it can occur due to the transient binding of a protein to an immobile structure within the cell. The simulations showed that the spatial accuracy of the particle tracking generally does not limit the detection of such binding events. However, changes in mobility can only be detected reliably when they last for a sufficient number of frames.

Having established the possibilities and limitations of single-particle tracking of gold nanorods in vitro, a next step is to move in vivo. In Chapter 3 we tested delivery of gold nanorods in live cells with different techniques: incubation, electroporation, cell-squeezing and single-cell microinjection in HeLa cells and COS1 cells. We also tested injection in the yolk of zebrafish embryo cells. For each technique we evaluated the delivery efficiency and the short-term consequences for cell viability. When the delivery of gold nanorods was successful, we analyzed their mobility by mean squared displacement analysis. We found three populations of nanorods: immobile, freely diffusing and diffusing within a confinement. In zebrafish embryo cells all the mobile rods were freely diffusing. In HeLa cells the diffusing rods were about 50% and in COS1 cells about 70% of the total. The diffusion coefficients were around 0.006 $\mu m^2/s$, lower than the expected values for unrestricted diffusion, but compatible with our previous findings. The confinement radius was around 0.3 $\mu m$. The mobility parameters we obtained were the same in cytoplasm and nucleus of HeLa cells.

Functionalization of gold nanorods is a necessary step for targeted delivery and for their use as labels to follow selected proteins in cells. Chapter 4 describes the functionalization of gold nanorods with nuclear localization signal. These peptides signal transport of biomolecules to the cell nucleus. Single-cell microinjection was used to deliver gold
nanorods in live HeLa cells. The efficiency of microinjection showed some variability between experiments and influenced the nuclear targeting results. Nevertheless, we observed a significant increase of nuclear localization of gold nanorods when functionalized with the nuclear localization signal. The nuclear targeting efficiency was around 15%, and was probably limited by the size of the particles. We noticed clearance of gold nanorods from the cytoplasm over time, for both functionalized and non-functionalized particles. The mobility of gold nanorods did not depend on their functionalization. Overall, these experiments showed that gold nanorods functionalization with nuclear localization signal can be used for nuclear targeting, but only with a small yield.

When performing a single-particle tracking experiment, the possibility to verify whether the particles are bound to the biomolecules of interest can be of great help. Gold nanorods have sensing capabilities that are particularly precious for this aim. The position of their plasmon spectrum shifts in response to changes in the dielectric constant of the environment, caused for example by the interaction with biomolecules. Using single gold nanorods, the shift in the spectrum peak can be used to detect low concentrations of ligands, down to the single-molecule level. Two-photon excitation spectra have been shown to exhibit narrower peaks than one-photon or scattering spectra, and a peak shift can be measured with higher accuracy. In Chapter 5 we explored the possibility to perform two-photon excitation spectroscopy with our multifocal scanning microscope, testing different gold nanorod samples. The spectra showed unexpected complexity, apparently unrelated to the sample features. To explain the origin of such spectra, we first verified the two-photon nature of the excitation produced in the sample. Then, we checked for the presence of gold nanorod clusters, by correlating the spectra to electron microscopy images of the sample. Finally, we characterized the response of different elements in the setup to variations in the excitation wavelength. Our results showed that the modulations in excitation power, while scanning the wavelength, strongly correlate with the shape of the spectra. We pinpointed the origin of these modulations to some elements in the setup. By removing these elements we could later obtain spectra of single gold nanorods.

The use of gold nanorods for in vivo single-particle tracking proved
to be challenging, mainly due to the large size of the particles and their stickiness before and after functionalization. In our work, we chose particles with sizes ranging from 10 nm x 40 nm to about 20 nm x 60 nm to favor high brightness and consequently high localization and tracking accuracy. This choice brought the disadvantage of slower diffusion and less efficient nuclear targeting when functionalized with nuclear localization signal peptides. However, we observed an increased stickiness for nanorods of smaller sizes. To fully exploit the promises of gold nanorods, a better compromise must be reached between size and brightness. To this aim, it would be interesting to study the fate of gold nanorods in cells as a function of their size.

Nevertheless, our results are encouraging. Efficient delivery of gold nanorods inside living cells was obtained through single-cell microinjection, electroporation and injection in the yolk of zebrafish embryo cells, with limited effect on cell viability. The mobility results that we obtained for gold nanorods in different cells were comparable, but quite different from the mobility we observed in glycerol. Successful functionalization of gold nanorods with nuclear localization signal peptides was proved by nuclear localization. Moreover, we illustrated the possibility to detect changes of mobility within gold nanorod traces and established the limitations of such analysis.

The successful delivery and functionalization of gold nanorods are the first steps to a number of possible experiments inside living cells. Nanometer-accuracy single-particle tracking of gold nanorods functionalized with proteins may give detailed insight into the dynamics of proteins. For example, gold nanorods can be functionalized to bind to nuclear proteins or to specific DNA sequences to follow protein-DNA interactions in the cell nucleus for longer time and with higher spatial resolution compared to other labels. Conjugation of gold nanorods to transcription factors (such as the glucocorticoid receptor) could reveal new mechanistic details of transcription regulation and on the fate of the receptor after this process. Gold nanorods bound to DNA sequences could also be used to study chromatin condensation.

The high potential of two-photon microscopy of gold nanorods must be further investigated before it can be used for sensing experiments. We encountered challenges in acquiring spectra of single gold nanorods, due to imperfect spectral properties of our setup. Recently, removing the polarization elements in the setup yielded better spectra. With this
advancement, the spectra of single gold nanorods may prove to be sensitive to the interaction with molecules. This opens up new possibilities, such as single-molecule sensing in live cells.