**Reply to Reviewers’ Comments**

We appreciate the in-depth and thoughtful reviews of all three reviewers. Below, we answer individually the raised concerns and comments, pointing out the modifications made to the text. The three reviewers expressed concerns regarding the heating of the nanoparticles as they could damage the cells. These concerns are answered collectively in the answer to Reviewer #3, as well as individually for each Reviewer.

We have marked in bold all the modifications included in the text, and strove explicitly to state in our answer where those modifications were done.

**Peer Reviewer #1:**

In this paper the authors describe the use of anti-Stokes emission of imaging gold nanorods even in the presence of a high fluorescence background. This is an interesting technical advance that will be viewed with interest by some readers. There are a few points of clarification and additional experiments that are required before the paper is ready for publication:

1. The authors should more clearly define what they mean by luminescence, and explain how it differs from scattering in the specific case of gold particles.

*Author reply: To make a clear distinction between scattering and luminescence we have modified the 5th paragraph of the introduction; now it clearly states what we refer to when mentioning luminescence. Luminescence arises from the radiative recombination of electron and holes in the metal; it is possible, however, that the emission has exactly the same energy than the excitation and therefore would be indistinguishable from elastic scattering. However, the filters needed to prevent excitation light from reaching the detectors block this portion of the spectrum. Therefore, (red- or blue-shifted) luminescence is necessarily different from scattering in our experiments.*

2. The authors should show the absorption spectra of the gold nanorods used here.

*Author reply: The extinction spectrum of the rods used in this work is presented in the Supplementary material, Figure S2, together with a TEM image of the particles, in Figure S4.*

3. If possible, the authors should estimate the approximate quantum yield for anti-Stokes luminescence for the particles used here. I was curious to know it, at least to an order of magnitude, and I'm sure other readers would like to know this too.

*Author reply: To answer this point, we have included a paragraph in the text with a short discussion regarding the quantum yield of the anti-Stokes emission. It has to be noted that this paragraph is slightly speculative, as we didn't perform single-particle absorption characterization and the reported quantum yields of particles normally rely on exciting off resonance. See for instance the work of Yorulmaz et al. Nano Lett. 2012, 12, 4385−4391.*

4. Figure 3 was hard for me to interpret, since it describes Stokes and anti-Stokes, but has emission both above and below the excitation wavelength in both cases. This does not accord with my understanding of Stokes and anti-Stokes, which is defined relative to the absorption or loss of vibrational quanta rather than by the wavelength of the emission relative to the absorption maximum. It's quite possible that my understanding is incorrect, but in any case the authors should clearly define what they mean here, and clarify this point for readers like myself.

*Author reply: Figure 3 represents the total anti-Stokes (a) and Stokes (b) emissions exactly as defined by the reviewer; the horizontal axis is the position of the resonance of each particle and not the energy of the emission. To make it clearer, we have modified the caption of the figure and included a comment in the main text. It now reads: “The anti-Stokes emission was calculated by integrating the recorded spectra at wavelengths shorter than the excitation laser, while the opposite was done for the Stokes. This can also be directly measured by recording the emission intensity after a short-pass or long-pass filter.”*

5. Does the illumination intensity used here cause local heating?

*Author reply: The illumination indeed causes local heating. The same concern was raised by the three reviewers and is now addressed in the main text in the last paragraphs of the discussion. Moreover a viability test was performed in order to ensure that even after irradiating the nanoparticles in the vicinity of the cells they show no damage. Please refer to the answer to Reviewer #3 for further clarification.*

6. The authors should measure quite a few more particles (n>10) for figure 6. 2 is not at all sufficient given the variability in particle properties (see fig.3).

*Author reply: To address this concern, we have added a new Figure to the Supplementary Material (Fig. S8), and we mention it in the main text when discussing Figure 6. We show the signal-to-background ratio for more than 10 particles in each case: Stokes and anti-Stokes under non-stained cells and anti-Stokes under stained cells.*

7. The experiment using ATTO 647N to provide background fluorescence is nice (Fig. 5 and 6), but is not analyzed in sufficient detail. The authors should provide an image of the labeled cells so that the reader can see if the dye is uniformly labeling the membrane. Also, the authors should more clearly define the dark counts, background counts in the presence of the cell but no dye, and counts resulting from the dye for both the Stokes and anti-Stokes measurement.

*Author reply: Part of these remarks were already present in the main text but maybe not sufficiently clear. We have therefore updated the text according to the suggestions of the reviewer. We now explicitly give the count rate of the background in the presence of cells with and without dye for both measurements.*

*Figure S7 shows a 20×20 μm2 scan of the stained cells where it is possible to observe the background signal. As stated in the text, we have incubated the cells with ATTO 647N, thereby staining not only the cell membrane but also the cytoplasm. Since this was a non-specific staining, we couldn't prevent the formation of regions with higher densities of dye and this appears as the places where a higher count rate is observed (for instance in the top left part of the image and in the bottom left.)*

8. No imaging technique that I am aware of is literally background free. The title should be changed in this regard.

*Author reply: The title has been changed to “Background suppression in imaging gold nanorods through detection of anti-Stokes emission”.*

**Peer Reviewer #2:**

Carattino et al. describe the use of anti-Stokes detection of nanorod emission for background free imaging even in the presence of alternative fluorescence sources. The technique is potentially useful for multimodal imaging, enabling for example single particle tracking together with larger field of view imaging. The approach is demonstrated with a confocal microscope, but should be usable with any fluorescence microscope. Impressively, they show that even in the presence of cells containing fluorescent dyes excited by the same illumination wavelength, they can still clearly identify the nanorod emitters. The work is interesting, novel and potentially useful for a wide audience and I therefore recommend publication, after minor revisions suggested below.

Major Comments:

1. The abstract states that: "we show that even in a cell containing the fluorescent dye ATTO 647N, the signal-to-background ratio of the anti-Stokes emission is higher than 10" and in the conclusion "this work shows that the technique can be easily extended to imaging in fixed cells, in vivo or even for tracking particles in real time"; but as they state themselves, no imaging inside a cell or even technically in the cell membrane is presented. The wording should thus be adjusted to more accurately reflect what actually has been done and what can be potentially done in the future.

*Author reply: We agree with the comments of the reviewer; the abstract and the conclusions have been changed to reflect this.*

2. The technique relies on illuminating the gold nanorods on resonance but makes no mention of local heating as a result of absorption that could be a problem for imaging in cells. It is stated that one cannot illuminate above 53 kW/cm2 because the rods reshape, and that the luminescence quantum yield is on the order of 10-6, but no mention is made as to what happens for illumination powers on the order of 15-30 kW/cm2, which is the range that seems to be predominantly used. If 53 kW/cm2 causes effectively melting of a metallic rod, I am concerned what would happen to cellular material at only half that illumination power. This should be clarified to ensure that sufficient Anti-Stokes photon flux is achievable even in a realistic imaging environment.

*Author reply: Heating of particles is indeed a major concern for imaging purposes. Firstly we would like to point out that the reshaping of the particles happens at lower temperatures than melting of gold. Reshaping is caused by thermally activated surface diffusion of gold atoms, leading to sphere-like particles. For small rods, it starts already at temperatures as low as 100 °C, over times of minutes; gold melting is a phase transition at around 1000 °C. This difference, for instance, allows to excite gold spheres with much higher powers than nanorods. For further clarifications in this point, please refer to the answer the Reviewer #3.*

Minor Comments/Clarifications:

1. The labelling of figures is confusing in that reference to figures in the main text and supplementary information is not clearly separated. On page 3 reference to Figure 1 implies the first figure in the main text, while on page 6 Figure 1b refers to the first figure in the supplementary/methods section.

*Author reply: This has been corrected. Figures in the Supplementary Material are now labelled with a preceding S.*

2. In the results section at the top of page 10, the sentence "In both cases the irradiation intensity was kept at 30 kW/cm2 in the back aperture of the objective" is unclear to me. I assume they mean that the intensity was 30 kW/cm2 at the sample, because 30 kW/cm2 in the back aperture being focused to a spot on the would be an enormous amount of power.

*Author reply: Indeed, it was a mistake in the text that has now been corrected. The power was 30 kW/cm2 at the sample.*

3. It would have been nice to see what the images/contrast look like when imaged onto a camera, to see how the signal-to-background ratio looks without the help of confocal imaging, as widefield imaging is mentioned as a potential alternative.

*Author reply: We agree that it would have been a nice addition to the paper to show a widefield image. However at the moment of the experiments no camera was available. We may work on this for a later experiment.*

**Peer Reviewer #3:**

In the manuscript "Background-free imaging of gold nanorods through detection of anti-Stokes emission" the authors describe an elegant and simple method to image gold nanorods within cells. This novel contrast agent has many unique and useful features compared to dyes.

The physical part of the work is rigorous and well described. It seems necessary that for the intended biological applications, the authors address the issue of damage to the cells with some more detail.

However, if this is convincingly answered, the work is a \*very\* important breakthrough in cell imaging. Having a new class of contrast agents will lead to novel insights in many fields. I would therefore strongly support timely publication of this work in this journal.

Detailed comments:

1. The laser power densities used in this work (in the order of kW/cm2) could potentially lead to high temperatures if the nanoparticles - as sometimes used in Plasmonic Photothermal Therapy (PPTT). In addition, photogenerated singlet oxygen species photodynamic therapy) could be an issue. Ideally, the authors would either show cell viability after imaging by some standart test (for example a simple alamar blue test)- or show convincing theoretical arguments why those problems should not arise.

*Author reply: Indeed the concern about the temperatures reached by the particles has been raised by the all the reviewers. To address this questions we have included two paragraphs at the end of the discussion in which we argue that the dissipated power by a single nanoparticle is several orders of magnitude lower than those reported for Photothermal Therapy. See for instance*

1. O’Neal, D. P., Hirsch, L. R., Halas, N. J., Payne, J. D. & West, J. L. Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. *Cancer Lett.* **209,** 171–176 (2004).
2. *Huang, X., Jain, P. K., El-sayed, I. H. & El-sayed, M. A. Determination of the Minimum Temperature Required for Selective Photothermal Destruction of Cancer Cells with the Use of lmmunotargeted Gold Nanoparticles. Photochem. Photobiol. 412–417 (2006). doi:10.1562/2005-12-14-RA-754*

*Moreover it is possible to calculate the temperature of the particle under standard irradiation conditions and see that the surface increases 25 °C, while in the same paper of Huang et Al. they mention temperatures of 70 °C to induce cell death and O’Neal et al. shows an average temperature of at least 50 °C. In our case the increase is limited to the vicinity of one particle; 50 nm away from the surface the temperature increase is no higher than 5 °C.*

*Finally we have performed a viability test of the cells after the imaging procedure and see no difference with those which were not irradiated. This figure was added to the supplementary material and is referred to in the results of the main text.*

2. Did you observe NP uptake?

*Author reply: Because of how the samples are prepared, it is not possible to observe NP uptake. Gold nanorods are fixed to the glass substrate to avoid their diffusion out of focus. A more detailed explanation on the sample preparation can be found, for instance in: Zijlstra, P. & Orrit, M. Single metal nanoparticles: optical detection, spectroscopy and applications. Reports Prog. Phys. 74, 106401 (2011).*

3. Could you show overview pictures of the cells with lower magnification (e.g. 20x or 40x) to compare irradiated and non-irradiated areas?

*Author reply: These images have been included in the supplementary material, together with viability tests. There is no visible difference between the irradiated and non-irradiated regions.*

4. Please provide details on laser spot size, laser fluence and type (pulsed, cw laser) prominently in the main text

*Author reply: In this work we employed two CW lasers (532nm and 633nm). We have now explicitly stated in the experimental section that they are continuous and not pulsed. Both lasers used were focused to a diffraction-limited spot through a high-NA objective. This was now clearly stated in the text.*