JoVE submission JoVE58389 – Surface-enhanced resonance Raman scattering nanoprobe ratiometry for the detection of microscopic ovarian cancer via folate receptor targeting – Response to editorial and peer reviewer comments.

We would like to thank the editorial staff and the reviewers for their constructive comments. We have addressed the concerns and requirements to the best of our abilities, resulting to an improved manuscript.

**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.  
2. Please remove the embedded figure(s) and figure legends from the manuscript. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.  
3. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.  
4. Figure 2: There are both “x 100” and “x 1,000” labels in the figure. Is “x 1,000” part of the y-axis scale label? Please clarify or move “x 1,000” to the left.  
5. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”  
6. Please rephrase the Abstract to more clearly state the goal of the protocol. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.  
7. Please rephrase the Introduction to include a clear statement of the overall goal of this method.  
8. Please define all abbreviations before use.  
9. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.  
10. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.  
For example: Amicon Ultra, CAS: 99126-64-4, Renishaw InVia, H&E, MATLAB (v2014b), PLS Toolbox (v8.0), etc.  
11. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.  
12. 3.1: Please clarify when thiol is added and specify the amount of thiol added. How long is the ultrasonication done?  
13. 5.3: Please specify all surgical instruments used. How large is the incision?  
14. Lines 227-229: Please add these as a step or sub-step.  
15. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.  
16. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.  
17. Please discuss any limitations of the technique.  
18. References: Please do not abbreviate journal titles.  
19. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
Minor Concerns:  
The report is well written and the protocol is clearly explained. The results support the conclusion. I have comments related to the terminology used in the manuscript.  
SERS and SERRS are well established techniques based on localized plasmon enhancement using metallic nanostructures. In the manuscript the authors use the terminology: SERS nanoparticles and 'surface enhanced resonance Raman scattering' (SERRS) nanoparticles. The addition of "nanoparticles" is unnecessary and confusing.  
On page 3 you read: "molecularly targeted nanoparticle provides the specific signal, and a non-targeted control nanoparticle, with different Raman spectrum, accounts for non-specific background". Raman spectra are collected from molecules not nanoparticles.  
The confusion persist when explaining the experiments: "Two distinct "flavors" of Raman nanoparticles are synthesized, each deriving its fingerprint from a different organic dye." In fact, the authors are using dye-embedded core-shell nanoparticles for surface Raman enhancement (spectroscopic tags). For these special nanoparticles, the term "SERRS nanoprobe" (dye-embedded core-shell nanoparticles tailored for surface Raman enhancement) used later in the manuscript is adequate and avoid misunderstandings.

We thank the reviewer for pointing out this confusing terminology. We have edited the manuscript for more consistent use of the terms. We now use the term “nanoprobe” for the final construct (with antibody or PEG), and the term “nanoparticle” for the previous steps of synthesis (stars, silicated, and thiolated).  
  
  
**Reviewer #2:**  
In this manuscript, the authors provide a protocol to image ovarian cancer by SERS tags. The protocol includes: nanostar synthesis, silica formation, surface functionalization, mouse model, nanoparticle injection/imaging, data processing/visualization.  
The protocol is definitely clear for the readers already working in the Rama tags field, especially for the tumor labelling. To broaden the readership, I suggest the authors add a figure to illustrate the "surface functionalization" because the readers in the related fields may be interested in the protocol but not familiar with those surface chemistry processes.

We have edited Figure 1 to include an extra step for the surface functionalization, and edited the caption accordingly.

In addition, I found that in this manuscript the recent related works cited (ref 21 to re 32) are all from the same journal (Nanotheranostics). Also, it is not critical but I would suggest authors also list other highly related works recently published in other journals.

We regret this oversight. We have added references from other journals.  
  
  
**Reviewer #3:**  
Manuscript Summary:  
The authors propose to use SERRS spectroscopy on plasmonic core-shell particles targeting the most over-expressed membrane receptor in mouse ovarian cancer cells. The work is convincing and rather well written.  
Some aspects deserve to be clarified before publishing the work  
  
Major Concerns:  
- The introduction must be more informative about the possibility of discriminating between single cells in a tumor and not by SERS. A preliminary in vitro study would have helped to rationalize the level of non-specific interaction of the nanoprobe. Cite and discuss the following work in the introduction section:  
Fasolato C, et al. Folate-based single cell screening using surface enhanced Raman microimaging.  
Nanoscale. 2016, 8, 17304.

We have added the related reference in the introduction.

- The authors report: "Typically, the size (hydrodynamic diameter) of the gold nanostar core is expected to be around 80 nm, and the silica shell is around 20 nm thick, making the total nanoparticle size around 120 nm" please explain.

The nanoparticle star-shaped gold core has a hydrodynamic diameter, as determined by DLS (Z-average) or nanoparticle tracking analysis, typically in the range of 80 nm. Once a spherical shell is formed around the core, approximately 20 nm in thickness (all around the core), the total diameter of the particle is 120 nm. This can be seen in the TEM images provided in Figure 2.

- Figure 3: The authors report the sentence "Absorbance maxima at lower wavelengths are a sign of either spherical morphology or aggregation." please explain. Why do you expect aggregation of nano-particles to cause a blue instead of a red shift?

As correctly pointed out by the reviewer, aggregation of spherical nanoparticles typically causes a bathochromic (“red”) shift of the plasmon resonance. Our particles have resonance in the red light region due to their star-shaped morphology. Aggregated nanostars give rise to less defined (more spherical) morphologies with plasmon resonance in the visible region, causing a hypsochromic (“blue”) shift.

- The author declare "After baseline subtraction, the fluorescence band is removed, the Raman peaks become prominent" florescence of what, please specify. Estimate the SERS gain factor by showing also the Raman dye control only.

As shown in the Figure 3, the raw spectra collected have a strong fluorescence band (the wide gaussian curve) on which the Raman bands are superimposed. The fluorescence mostly derives from the IR fluorescent dyes we used as Raman reporters. By using fluorescent dyes that absorb in the excitation wavelength, it is possible to achieve surface enhanced **resonance** Raman scattering, further enhancing the nanoprobe signal over other molecules that do not absorb the excitation light. Although fluorescence in the vicinity of the gold nanoparticle is quenched for the most part, it is our understanding that residual dye molecules within the silica shell (several nm away from the gold) give rise to this fluorescent background. Unfortunately, the Raman signal of the free dye (without plasmonic enhancement) is negligible compared to the molecule’s fluorescence, and we were not able to capture it without saturating our detector.

Specify with respect to what the spectra shown in figure 3 have been normalized. Specify in the methods the Raman calibration with respect to the grating used.

The spectra were normalized to have unit area (area under the curve = 1), as stated in the figure caption and also in Step 6.2. Calibration of the Raman scanner should be done according to the manufacturer’s recommendation, and is typically performed using a common standard (e.g. a silicon wafer). We have added this recommendation to the text, in the discussion section.  
  
Minor Concerns:  
- in the final section of the work discuss whether such nanoprobe once targeted on the tumor might be employed also as a valuable therapeutic or theranostic tool.

We have added a paragraph discussing the theranostic potential of the probe in the discussion section.  
  
  
**Reviewer #4:**  
Manuscript Summary:  
The manuscript describes a protocol for SERRS nanoparticle imaging for surgical tumor resection in small animal models. The protocol covers the chemistry behind fabrication and functionalization of the nanostar SERRS agents, their characterization in terms of successful manufacture. In addition, results are given for the use of the nano stars in an in vivo ovarian cancer small animal model. Ovarian cancer cells were injected in the peritoneum of immunocompromised mouse models, which constitutively express bioluminescent markers enabling their location to be tracked with optical imaging. The nano star model used was a so-called ratiometric model, in which nanoparticles of one color are non-targeted, and nanoparticles of another color are targeted for the folate receptor alpha. The idea is that the non-targeted nanoparticles will differentiate signal from successfully targeted nanoparticles, accounting for non-specific nanoparticle binding.  
  
Major Concerns:  
The primary concern with the protocol is that the method does not clearly demonstrate that the ratiometric targeting method actually works to identify residual micro metastases in the mouse model. The authors state that even targeted nanoparticles "tend to adhere onto the visceral surfaces even in the absence of their target." If this is true, then it is not clear how having additional nanoparticles which also have nonspecific binding will account for this limitation. In other words, why are 2 non-specific labels better than 1 non-specific label?

The targeted probe binds preferentially on the folate receptor, which is overexpressed by the tumor, but also sticks on other surfaces that are not cancerous. It is, therefore, incorrect to call it “nonspecific”. However, when used on its own, it creates high background with many false-positive signals (Figure 4, middle left). The non-targeted probe coats the visceral surfaces in a similar way as the targeted-probe (Figure 4, middle right), but without increased accumulation in the areas of the folate receptor. By dividing the two signals, the non-specific adhesion is accounted for, and the increased accumulation in the areas of the overexpressed folate receptor is revealed.

The fact that the scheme may not work very well is demonstrated in Figure 4, where the location of tumor cells is indicated by the bioluminescence image, yet the thresholded ratio image does not seem to overlay the location of the bioluminescent (tumor) areas with any accuracy.

We regret that the reviewer feels that the accuracy is low. The perceived mismatch may be due differences between the two modalities (Raman vs bioluminescence imaging), the positioning of the animal related to the detector in each case, the dynamic range (sensitivity) of the detectors (102 for BLI, 104 for Raman), and focal plane of imaging. We would kindly refer to our related publication (doi:10.1021/acsnano.6b06796) where more extensive validation is performed.

In any case, no method to validate that the method actually works is provided. The method stops at simply describing how to display the image, but does not describe how the obtained images can be compared to ground truth (either from the bioluminescence imaging, or by sampling the cavity and doing pathology). This seems to be a major oversight or limitation of the protocol. If the authors could describe a method for validating the efficacy of the protocol, and also demonstrate that the protocol is efficacious, then it would be more suitable for publication in JOVE.  
We believe we have validated our system sufficiently for our specific application, as described in our related publication. However, we would be delighted if more validation methods, more model systems, and more imaging scenarios were explored by other groups using our method. This is the reason we are providing the protocol, in a generalized format, so the community can validate it on their own systems, with new targets and other tumor types.

Minor Concerns:  
There are some minor details that are missing or confusing. In Procedure 1.1 and 1.2, the gold solution is called different things (tetrachloroauric acid or gold trichloride). Whether the microscope used is upright or inverted is not specified, although the positioning of the animal in a supine position is prescribed.

We thank the reviewer for the observation, we have clarified these points in the text.