Published in final edited form as:

ACS Nano. 2017 February 28; 11(2): 1488–1497. doi:10.1021/acsnano.6b06796.

Folate-Targeted Surface-Enhanced Resonance Raman Scattering Nanoprobe Ratiometry for Detection of Microscopic Ovarian Cancer

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Abstract

Ovarian cancer has a unique pattern of metastatic spread, in that it initially spreads locally within the peritoneal cavity. This is in contrast to most other cancer types, which metastasize early on *via* the blood stream to distant sites. This unique behavior opens up an opportunity for local application of both therapeutic and imaging agents. Upon initial diagnosis, 75% of patients already present with diffuse peritoneal spread involving abdominal organs. Complete resection of all tumor implants has been shown to be a major factor for improved survival. Unfortunately, it is currently not possible for surgeons to visualize microscopic implants, impeding their removal and leading to tumor recurrences and poor outcomes in most patients. Thus, there is a great need for new intraoperative imaging techniques that can overcome this hurdle. We devised a method that employs folate receptor (FR)-targeted surface-enhanced resonance Raman scattering (SERRS) nanoparticles (NP), as folate receptors are typically overexpressed in ovarian cancer. We report a robust ratiometric imaging approach using anti-FR-SERRS-NPs (α FR-NPs) and non-targeted SERRS-NPs (nt-NPs) multiplexing. We term this method of ‘Topically Applied Surface Enhanced Resonance Raman Ratiometric Spectroscopy’ (“TAS3RS” (\tsasers\)) for short). TAS3RS successfully enabled the detection of tumor lesions in a murine model of human ovarian adenocarcinoma regardless of their size or localization. Tumors as small as 370 μ m were detected as confirmed by bioluminescence imaging and histological staining. TAS3RS holds promise for intraoperative detection of microscopic residual tumors and could reduce recurrence rates in ovarian cancer and other diseases with peritoneal spread.

Graphical abstract

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Author Contributions: AO developed the animal model, synthesized the nanoparticles and performed the imaging studies. CA developed the ratiometric algorithm and performed data analysis. AO and CA performed physical characterization of the nanoparticles. MW helped develop the concept and gave critical advice. MFK supervised the project. All authors contributed to discussions on the project. The manuscript was written by AO and CA and edited by MFK.

Supporting Information Available: Additional TEM images, spectral preprocessing, ratiometric approach, biodistribution and characterization of Folate Receptor expression. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

Keywords

Raman; SERS; Nanoparticle; Molecular Imaging; Ovarian Cancer

Ovarian cancer is the deadliest gynecologic malignancy, accounting for the death of 12,303 women in 2013 in the United States alone.¹ The overall poor prognosis can be explained by the fact that the majority of patients present at an advanced stage (FIGO stage III or IV), when the primary ovarian tumor has already disseminated throughout the abdominal cavity. Even the current gold standard of cytostatic chemotherapy (carboplatin and paclitaxel) results in a median overall survival of only 39 months after primary diagnosis.² Prevention of progression and recurrence remains a major hurdle in the management of ovarian cancer. The key factor for effective treatment is a complete resection of all visible lesions, improving the prognosis to up to 64 months of median overall survival.³ Attempts have been made to address the problem of residual undetected microscopic peritoneal disease by administering chemotherapy directly into the peritoneal cavity, which has shown a significant increase in overall survival.⁴ This can be attributed to an enhanced effect of cytostatic drugs on microscopic tumor residuals after removal of all visible lesions.⁵ However, not all tumor-implants respond to chemotherapy, resulting in recurrence in most patients. It is likely that a more thorough resection of those microscopic lesions would further improve the patients' prognoses, and this goal represents the motivation for this work.

The Folate Receptor (FR) is overexpressed in more than 70% of primary ovarian cancers and has been studied extensively for both targeted treatment⁶ and intraoperative imaging.⁷ In this study we have explored the ability of targeted Raman nanoparticles to delineate ovarian cancer implants on the peritoneum and visceral surfaces after intraperitoneal injection.

Raman imaging is an optical spectroscopic imaging technique that provides a highly specific spectral signature of the substance interrogated. Intrinsic Raman signals are relatively weak, therefore nanoparticle-based contrast agents have been developed using 'surface enhanced Raman scattering' (SERS). Such agents rely on plasmonic effects of gold nanoparticles to enhance the Raman signature of molecules adsorbed on the nanoparticle.^{8, 9} By using Raman reporter dyes and noble metal cores that are also in resonance with the excitation laser, 'surface enhanced resonance Raman scattering' (SERRS) nanoparticles (NPs) can achieve even greater signal enhancement, resulting in ultrahigh sensitivity with detection limits in the low femtomolar or even attomolar range using rapid *in vivo* imaging settings.^{10,11} Unlike methods based on fluorescence, SERRS-NP imaging does not suffer from autofluorescence. This increases the specificity of detection of SERRS-NPs as a

contrast agent, and has shown promise in a variety of cancer types (*e.g.* pancreatic, breast, prostate cancer, and sarcoma) in mouse models after intravenous injection.¹¹⁻¹³

However, when it comes to ovarian cancer, there exists a major limitation for an intravenous application of SERRS-NPs: the primary metastatic route of ovarian cancer does not occur through hematologic, but instead peritoneal spread.¹⁴ This involves selective invasion of the mesothelium of the peritoneal surface, forming micro-metastases without access to the vasculature. These microscopic implants are supplied mostly *via* local diffusion until a size of approximately 1 μm^2 ^{15,16} and are therefore more difficult to reach with a systemically injected nanoparticle agent.

We hypothesized that the local, intraperitoneal application of FR-targeted SERRS-NPs could visualize microscopic ovarian cancer metastases. This strategy could have wide-ranging applications in image-guided resection or minimally invasive destruction, and bypass the need for systemic NP injection. Relying on the local intraperitoneal injection of SERRS-NPs should also markedly increase the chance for FDA approval, because potential systemic side effects are minimized or eliminated.

Preliminary experiments of intraperitoneal SERRS-NP injections demonstrated very low tumor specificity as the SERRS-NPs would adhere indiscriminately on peritoneal or visceral surfaces and would also get trapped in anatomical crevices. Due to this non-specific adherence of SERRS-NPs we concluded that – regardless of the targeting moiety – we needed to account for a high background distribution of NPs. We hypothesized that targeted NPs would have a qualitatively similar distribution as non-targeted NPs, but an increased adherence to tumor tissue. While such behavior might at first seem problematic, ratiometric methods can aid greatly in clearly delineating the distribution of the receptor of interest due to specific binding of targeted NPs, as demonstrated *ex vivo* by others.^{17,18} Although folate would be the most logical targeting moiety, our initial experiments using folate-conjugated NPs did not sufficiently discriminate between healthy and malignant tissue, possibly due to the relatively neutral surface charge or low binding affinity of folate once attached to the NPs (data not shown). Thus, we opted for antibody-directed FR targeting, resulting in high specificity of our imaging scheme.

Results

Nanoparticle Synthesis and Characterization

First, we synthesized two different ‘flavors’ of SERRS nanoprobe with gold nanostar cores and a silica shell, as described in Methods: a targeted nanoprobe – functionalized with an anti-Folate-Receptor Antibody ($\alpha\text{FR-Ab}$) *via* a PEG-Maleimide-Succinimide crosslinker (Figure 1A) and using the infrared dye IR780 as the Raman reporter – henceforth referred to as $\alpha\text{FR-NP}$; and a non-targeted probe (nt-NP) – coated with PEG₅₀₀₀-Maleimide and featuring the IR140 infrared dye as the Raman reporter.

Quality control of the synthesis process was performed with TEM imaging (Figure 1B, Supplementary Figure S1) to evaluate the formation of the silica shell, and dynamic light scattering (DLS) to measure changes in diameter and zeta potential. The average diameter of

the nanostar cores was 78 nm with a polydispersity index () of 0.281, silicated NPs were 131 nm (=0.125) and after functionalization α FR-NPs were 164 nm on average (=0.120) and nt-NPs 164 nm (=0.080) (Figure 1C). The zeta potential was also used to confirm successful functionalization of the NPs (Figure 1D). Both the silication and functionalization had negligible influence on the plasmonic characteristics of the SERRS-NPs (Figure 1E). The wide absorbance band is an effect of the broad size distribution of the NPs, which in turn is due to asymmetry and variable length in the stars' protrusions. Despite this, there is sufficient absorbance at our excitation wavelength to produce robust SERRS signal. The average antibody load on the NPs was determined by a BCA protein assay and was found to be approximately 0.43 μ mol of antibody per 1 nmol of α FR-NPs.

Development of SERRS-NP ratiometry

The distinct SERRS spectra of the two probes are shown in Figure 2A. For calibration of our ratiometric Raman algorithm we devised an *in vitro* dilution model (Figure 2B, C). The overall concentration of both probes decreases from top to bottom starting at 300 pM to 300 fM, and each column contains a different ratio of α FR-NP to nt-NP, spanning 5 orders of magnitude (100:1 to 1:100) in decades. A Raman scan of the plate was acquired with a Raman Microscope (inVia, Renishaw, Hoffman Estates, IL, USA) equipped with a 785 nm diode laser at 100 mW laser power, in StreamLine high-speed acquisition mode. These settings were chosen as they have been used previously for *in vivo* scans.^{11, 19}

A direct classical least-squares (DCLS) model was developed to visualize the presence of the Raman signature of each of the SERRS-NPs using the reference spectra shown in Figure 2A. DCLS is a linear regression technique that helps extract useful information from multivariate data (such as Raman spectra) by comparing them to a set of references. In this way, complex Raman spectra emanating from mixtures of NP populations can be easily decomposed into their individual constituents when reference spectra from pure NP populations are used. To generate the model, the reference spectra were preprocessed by baseline subtraction, normalization by the maximum value, and finally taking the first-order derivative. The DCLS model was then applied to each spectrum from the scan (with the same preprocessing) in order to calculate the predicted scores for each point on each of the components (Supplementary Figure S2). The scores on the components corresponding to the two SERRS-NPs are shown in Figure 2B, with Score $_{\alpha$ FR shown in red and Scores $_{nt}$ in blue.

The DCLS model was able to quantify the relative signal of the two probes down to concentrations of 300:3 fM. To generate a ratiometric map, we calculated the logarithmic ratio $r = \log_{10}(\text{Scores}_{\alpha\text{FR}}/\text{Scores}_{nt})$ for each point, and displayed it on a diverging scale: blue indicating $r < 0$, and red $r > 0$ (Figure 2C). The ratiometric calculation was successful and performed reliably down to the lowest concentrations. This algorithm and color assignment remains consistent for all following Raman scans presented in this paper. More information about the calculation can be found in Supplementary Figure S3.

TAS3RS – ovarian cancer imaging

Intraperitoneal administration was found to prevent systemic uptake of the NPs. A biodistribution study (Supplementary Figure S4) was performed on healthy mice (n=5)

showing negligible uptake in organs of the RES (liver, spleen) which are known to have a high nanoparticle uptake after intravenous injection.¹⁹

To generate the tumor model, athymic nude mice were challenged intraperitoneally (i.p.) with 4×10^6 SKOV-3 cells transduced with luciferase and green fluorescent protein. Tumor growth was monitored *via* bioluminescence imaging (BLI). After 24-40 days, tumor bearing mice (n=3) and healthy control mice (n=4) were subjected to Raman imaging with ratiometric analysis.

The imaging procedure is described in detail in the Methods section and was identical for both tumor bearing and control mice. A mixture of the two SERRS-NPs (1 mL PBS, 300 pM each) was injected i.p. Twenty minutes later luciferin was injected retroorbitally. The abdominal cavity was incised, washed with 60 mL PBS, the entire abdomen exposed and bowel resected for a better overview on pelvic organs and peritoneum. The whole mouse was imaged with BLI. Regions of interest were scanned with the Raman microscope to assess the correlation of the ratiometric signal to the BLI map. Raman imaging settings were identical to the *in vitro* scans reported above. For simplicity, we term this method of ‘*Topically Applied Surface Enhanced Resonance Raman Ratiometric Spectroscopy*’ (“TAS3RS” (tasers) for short).

Regardless of the seemingly unspecific distribution of both nanoprobe individually within the peritoneal cavity (Figure 3, 2nd & 3rd rows), the ratiometric TAS3RS algorithm did not show any positive signal in the four healthy control mice (Figure 3, 4th row, left), whereas in the tumor bearing mice TAS3RS was able to delineate tumorous lesions identified by BLI (Figure 3, 1st & 4th row, right). Additionally we created an alternative simplified rendering of the TAS3RS map, as it could be applied for surgical guidance, which only depicts the cancer lesions in red and leaves benign areas transparent (Figure 3, 5th row). Due to distortion of the organ geometry occurring in histological processing of a whole mouse, a direct comparison of TAS3RS to histology was not feasible.

Validation of TAS3RS *via* correlation to histology

To facilitate a direct comparison of TAS3RS and BLI to histology, we modified the experimental procedure by introducing a rapid organ dissection onto glass slides (upper abdominal organs, pelvis with urogenital system and retroperitoneum) in a separate cohort of mice (n=5) (Figure 4A). All slides were imaged *via* BLI and could then be scanned using the Raman microscope. With this approach the samples could be fixed and embedded while maintaining their geometry and orientation.

All specimens that showed BLI positive foci (Figure 4B) were scanned with the Raman microscope and our algorithm was applied (Figure 4C). Compared to the previous whole abdomen images, the segmentation into small area scans allowed for higher resolution. Again we found that TAS3RS enabled excellent delineation of BLI positive lesions.

Specimens subjected to TAS3RS were subsequently embedded, sectioned, and stained with hematoxylin and eosin (H&E). Histology images were compared to BLI and TAS3RS maps. To capture most lesions in large multi-organ samples with highly uneven surfaces, sequential

tissue sections at depths of 250 μm , 500 μm , and 750 μm were obtained (Figure 4D). Despite the evaluation of several slices per specimen, a few implants that were detected by both BLI and Raman were not captured on H&E stained slides (Figure 4 B, C: yellow arrows). However, nearly all lesions that were detected by TAS3RS were confirmed by histologic analysis to represent tumor and thus to be true positive. In fact evaluating the shown specimen, we observed neither BLI- nor histologically-positive lesions that were not detected by TAS3RS regardless of the site of implantation, thus demonstrating a high sensitivity of our approach.

TAS3RS reliably detected lesions in all scanned multi-organ slides. Two further examples are presented in Figure 5. Additionally to upper abdominal disease (Figure 5: left column), which was present in all mice ($n=5$), one animal also showed extensive pelvic spread of ovarian cancer (Figure 5: right column). In both examples all BLI positive foci (Figure 5A) were detected by TAS3RS (Figure 5B). All BLI and TAS3RS positive lesions except one (yellow arrows) could be also confirmed histologically (Figure 5C).

Discussion

In this work we present an approach to address the challenging goal of identifying microscopic intraperitoneal spread of ovarian cancer. We devised a technology that allows the detection of microscopic metastatic lesions anywhere in the abdomen or pelvis using a local (intraperitoneal) injection of the imaging agent. There are only limited examples of intraperitoneal lavage approaches in the literature that use fluorescence based imaging and therefore suffer from both lower tumor to background ratio as well as photobleaching.²⁰ The technique, termed here TAS3RS, is based on ultra-sensitive SERRS nanoparticles that we have recently developed, and makes use of the ratiometric information resulting from the differential homing of anti-Folate receptor SERRS nanoparticles and non-targeted SERRS nanoparticles. Applying TAS3RS in a model of intraperitoneal ovarian cancer demonstrated that metastases anywhere in the abdomen could be detected with high accuracy as verified by BLI and histological correlation. Unlike fluorescence-based intraoperative imaging techniques that are being evaluated in clinical trials,⁷ the method presented here does not require systemic injection of the contrast agent.

There are several advantages to this approach: (1) the “Raman-Fingerprint” of each Raman probe has a high sensitivity and specificity¹¹ allowing for very sensitive ratiometric imaging of multiple differently functionalized NPs, and is significantly more photostable compared to current fluorochromes;¹⁹ (2) unique SERRS-NP spectra cannot originate from endogenous biomolecules, hence overcoming the major disadvantage of autofluorescence and the issue of false positive signals in fluorescence imaging; (3) sulfhydryl-modified SERRS-NPs can be loaded with any desired antibody (or other targeting moieties) *via* simple thiol-maleimide chemistry, potentially facilitating a large library of antibody-dye combinations; (4) a ratiometric imaging approach with built-in reference standards can quickly generate reproducible maps and compensate for batch-to-batch and injection-to-injection variation; (5) the inert composition of the nanoparticles consisting of gold and silica, with extensive toxicity studies published in the past;²¹ (6) high degree of biocompatibility due to our silication method that does not require toxic surface primers; and

finally, and perhaps most importantly, (7) TAS3RS works *via* local intraperitoneal injection, thus bypassing the toxicity concerns that have hindered the clinical translation of many nanoparticle probes that enter the systemic bloodstream.

SERRS-NPs and other nanoparticle-based imaging agents can be used for intravenous injection as they home to tumors due to the enhanced permeability and retention (EPR) effect,^{10, 12, 13, 22} with a sufficient accumulation – partly due to leaky blood vessels – that allows delineation in surgical scenarios as delicate as brain surgery.²³ In topical application, this selective uptake of NPs cannot be achieved. However, by combining multiple NPs with different affinities to tumor tissues, a ratiometric comparison of high-affinity to low-affinity NPs can be performed and thereby detect cancerous lesions as shown here by TAS3RS.

In addition, imaging with TAS3RS does not depend on arbitrary thresholds that can complicate the standardization of the medical image. Based on the preprocessing methodology we employed, the predicted score for pure NP populations is equal to unity for both components, regardless of their physical Raman intensity. Spectra emanating from mixed populations are assigned lower scores by the model based on the ratio of their spectral components (as seen in Figure 2C). Since the model was normalized in this way, the ratios of the scores are independent from the intensities of the individual Raman reporter, and any batch-to-batch variation of the probes can be accounted for by obtaining new reference spectra specific to that experiment. In this way, the ratiometric image remains robust between experiments.

We have tested TAS3RS in an ovarian cancer model in a way amenable to *in vivo* imaging to simulate the most challenging application for this imaging algorithm: (1) multiple organ surfaces with different adherence of NPs, (2) limited washing due to necessity of preservation of correct anatomical orientation, (3) large scan areas, (4) correlation with both BLI and histology for validation. We were able to show that TAS3RS can accurately delineate BLI positive lesions.

Technologies for intraoperative intraperitoneal chemotherapy perfusion are frequently used in patients with peritoneal spread.²⁴ These devices could easily be adapted for perfusion with our SERRS-NP suspension. With a handheld scanner, occult lesions could be detected and destroyed in an automated manner (*e.g.* Argonlaser), thus further reducing the postoperative tumor burden. An example of Raman guided resection was recently demonstrated in brain tumor models.^{22, 23}

Our algorithm could easily be implemented in a software package included with a dedicated clinical Raman imaging instrument in the future. We envision that with future hyperspectral Raman scanners, TAS3RS-positive signals would be overlaid onto the photographic white light video in real-time. Those images could then be projected with an augmented reality device directly into the field of vision of the surgeon. Such real-time Raman imaging devices for clinical application are currently still in the stage of development.²⁵⁻²⁹ Additionally, nanoparticles in general are expected to take more time for regulatory approval than small-molecule fluorescent dyes; however, the fact that no systemic injection is required may markedly accelerate the approval of our approach.

Conclusion

In summary, the approach presented here – using intraperitoneal application of two SERRS-NPs and ratiometric signal analysis – has demonstrated robust delineation of tumor deposits with microscopic precision in an ovarian cancer model. This principle should have a high potential for clinical translation given its high accuracy and, most importantly, its local application route without systemic uptake.

Methods

Materials

Unless otherwise noted, all chemicals were obtained from Sigma Aldrich (St Louis, MO, USA).

SERRS-NP Synthesis

SERRS nanoparticles were synthesized as previously described,^{10, 11} with the modification that an additional Raman reporter (IR140) was used. Gold nanostars were synthesized by rapid addition of 10 mL of 20 mM HAuCl₄ to 1000 mL of 40 mM ascorbic acid at 4 °C under constant stirring. The synthesized gold nanoparticles were collected by centrifugation and dialyzed against deionized water (18.2 MΩ-cm; MWCO 3.5 kDa) for 3 days. The dialyzed gold nanoparticle dispersion (2.2 mL; 1.0 nM) was added to 18 mL absolute ethanol containing 360 μL 28% (v/v) ammonium hydroxide, 450 μL of 99.999% tetraethyl orthosilicate (TEOS), and 36 μL of either IR140 perchlorate (IR140) or IR780 perchlorate (IR780) at 25 mM in N,N-dimethylformamide under vigorous stirring. After 37 min, the reaction was quenched by the addition of 35 mL of ethanol, SERRS-NPs were collected after centrifugation, washed with absolute ethanol four times and redispersed in 850 μL ethanol. For sulfhydryl-modification 100 μL of (3-mercaptopropyl)trimethoxysilane (MPTMS) and 50 μL of deionized water were added and the solution was subsequently heated at 70 °C for 2 h. Next, SERRS-NPs were washed repeatedly first with ethanol and subsequently with 10 mM 2-(N-morpholino)ethanesulfonic acid buffer (MES pH 7.1). After the last wash SERRS-NP concentration was brought to 3-4 nM for further functionalization.

SERRS-NP Functionalization

The ab3361 (Anti-Folate Binding Protein antibody [LK26]) Ab clone was purchased from Abcam (Cambridge; UK) at a concentration of 0.4 mg/mL. In a first step, antibodies were incubated with 10-fold molar excess of a PEG-crosslinker (Poly(ethylene glycol)(N-hydroxysuccinimide 5-pentanoate) ether N'-(3-maleimidopropionyl) aminoethane (CAS: 851040-94-3; Sigma Aldrich; Lot: MKBK3123V) in MES buffer (pH 7.1) for 30 min. Amicon Ultra 0.5 mL centrifugal filters (Millipore, Darmstadt; Germany) with a MWCO 100 kDa were used to remove excess unreacted crosslinker and concentrate Abs. In a second step, sulfhydryl-modified SERRS nanoparticles 'IR780' were incubated with the antibody-PEG conjugates for 2 h at room temperature in 10 mM MES buffer (pH 7.1), at an Ab concentration of 200 μg/mL during reaction, to yield αFR-NPs.

Sulfhydryl-modified SERRS nanoparticles 'IR140' were mixed with an equal volume of MES buffer (pH 7.1) containing 2% (w/v) methoxy-terminated (m)PEG₅₀₀₀-maleimide (CAS:99126-64-4; Sigma Aldrich; Lot: BCBF3520V) and were allowed to react for 2 h at room temperature to produce nt-NPs.

Both α FR-NPs and nt-NPs were washed twice with 10 mM MES buffer (pH 7.1) and resuspended to a concentration of 600 pM.

SERRS-NP Characterization

Nanoparticles were placed on carbon film-coated copper grids (Ted Pella, Inc.) and images acquired with a transmission electron microscope JEOL 1200 EX (Peabody, MA) operating at 80 keV. Concentration and size distribution were determined by nanoparticle tracking analysis (NTA; NanoSight, NS500; Malvern Instruments Inc.; Westborough, MA). DLS size distribution (Z-average size) and zeta potential were measured with a Malvern Zetasizer in 10 mM MES buffer (pH 7.1). The antibody load of the α FR-NPs was determined with a BCA assay (Pierce™ BCA Protein Assay Kit (ThermoFisher)). The BCA reactions for the NPs as well as the BSA standards were performed in 1.5 mL tubes following the procedure provided by the manufacturer. After incubation, the tubes were centrifuged to precipitate the NPs, and 200 μ L of supernatant from each tube were transferred to a 96-well plate and scanned using a plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA).

Cell Line

The luciferase and green fluorescent protein transfected human ovarian adenocarcinoma cell line SKOV-3 Luc+/GFP+ was kindly supplied by Dr. Aras (MSKCC). Cells were cultured in full RPMI with 10% FCS (fetal calf serum) and passaged twice a week. For injection, cells were detached with 0.25% trypsin / 0.05% EDTA at 3 min incubation time and subsequently washed and resuspended in PBS for intraperitoneal injection.

To evaluate the expression of FR (also referred to as Folate Binding Protein) a Western blot was performed (Supplementary Figure S5). The KB (squamous cancer) cell line as a high FR expression control and SKBr-3 (breast cancer) cell line with low FR expression were purchased from ATCC. Cells were cultured in 75 cm² flasks and lysed at 80% confluence. The protein lysate was quantified with Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and a loading amount of 30 μ g was used. Anti-Folate antibody (ab3361 from Abcam, Cambridge; UK) was used at 1:1000 dilution (stock solution 0.4 mg/mL). Both low expression in SKBr-3 cells as well as overexpression in KB cells were confirmed. SKOV-3 cells showed a moderate but significant expression, indicating that this tumor model could be used for FR targeting *in vivo*.

Mouse Model

All animal studies were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center (#06-07-011). For imaging studies, athymic female mice (Nu/J mice; Stock-No: 002019) 6-8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Tumor cells (4×10^6 /mouse) were injected intraperitoneally in a volume of 200 μ L PBS. Tumor growth was monitored weekly by BLI

after retroorbital injection of 2 mg Beetle Luciferin (Beetle Luciferin / Potassium salt, Promega, Madison, WI, USA) in 50 μ L in an IVIS Spectrum (PerkinElmer, Waltham, MA). After 24-40 days disseminated peritoneal spread was detectable and mice were assigned to further experiments. For the biodistribution studies female C57BL/6J (stock #: 000664) 6-8 weeks of age from Jackson Laboratory were used.

Imaging Procedure

α FR-NPs and nt-NPs were mixed in a 1:1 ratio, resulting in a final concentration of 300 pM of each type. The SERRS-NP mix in MES buffer (1 mL, pH 7.1) was injected intraperitoneally. Twenty minutes later a retroorbital injection of 2 mg Beetle Luciferin in 50 μ L buffer was performed. After 5 min the mouse was sacrificed *via* carbon dioxide asphyxiation, the peritoneal cavity was exposed with a large sagittal incision and washed with 60 mL PBS. The intestines were resected with a ligation of the mesenteric vessels to reduce blood spill into the abdominal cavity. For whole abdomen imaging, mice with exposed abdomen were imaged with an IVIS Spectrum (PerkinElmer, Waltham, MA) within 20 min after luciferin injection. Mice were then transferred to the Raman Microscope (inVia, Renishaw, Hoffman Estates, IL, USA) equipped with a 300 mW 785 nm diode laser and scanned *ex vivo* with 100 mW laser power. The StreamLine high-speed acquisition mode was used for scanning, where multiple spectra are acquired under continuous laser illumination with the microscope stage constantly moving in straight lines. The spatial resolution of the scan was set to 14.2 μ m along the lines and 200 μ m across. Each point is illuminated for less than 100 ms. No averaging was performed during acquisition. Spectra were collected through a 5 $\times$ objective (Leica, Buffalo Grove, IL). Although the absorbance maximum of the NPs is in the 650 nm region, a 785 nm laser was used as this wavelength has a higher penetration depth and lower absorbance by biological tissues. Additionally, the dyes used were chosen to be in resonance with this wavelength, as to achieve the high SERRS signals reported here. For imaging-histology correlation experiments mice were dissected into two regions of interest after surgical exposure of the abdomen: upper abdomen (spleen, liver, pancreas, and stomach) and pelvis (uterus, ovaries, kidneys, bladder, and dorsal peritoneum). The multi-organ specimens were placed on glass-slides and remained unchanged for all scans and PFA fixation to preserve the exact anatomical orientation. Slides were first imaged using BLI in the IVIS Spectrum, and those specimens that showed BLI signal were subsequently scanned with the Raman microscope to assess correlation.

Histology

After imaging, multi-organ specimens were fixed in 4% PFA (MP Chemicals, Solon, OH) overnight at 4 $^{\circ}$ C, followed by a rinse with PBS for 15 min, and then kept in 70% ethanol until embedding in paraffin. Five micrometer thick sections were cut from the paraffin block and stained with hematoxylin and eosin and scanned with a Mirax digital slide scanner (Zeiss, Jena, Germany) for histopathological analysis. The slides were analyzed with Panoramic Viewer software (3DHistech, Hungary).

Biodistribution

Five wild type control mice were injected intraperitoneally with the TAS3RS cocktail and euthanized one hour after injection. Blood, liver, and spleen samples were collected. Tissue

samples were extracted with a biopsy punch. Specimens were weighed and 1 μL of MES was added per μg of tissue weight to normalize for size differences of the specimens. Organs were homogenized and 80 μL of each was transferred into a 384-well plate. Raman imaging was performed using scanning parameters identical to the other scans. The spectra collected from each well were averaged to obtain a representative spectrum from each tissue. These weighted intensities were used to determine the relative concentration of each SERRS-NP in blood and tissue samples.

Image Data Processing

All data analysis was performed in MATLAB (R2014b) and PLS Toolbox v.8.0 (Eigenvector Research, Inc., Wenatchee, WA). The reference spectra (such as the ones shown in Figure 2A) were obtained from pure (not mixed) NP suspensions, with each NP having the same concentration as the injectate. To generate the DCLS model, the reference spectra were preprocessed by baseline subtraction using a Whittaker filter (with width $\lambda=200\text{ cm}^{-1}$), subjected to L^1 -norm (normalization by the area), and followed by a Savitzky–Golay derivative filter (second degree polynomial fit, first-order derivative, width = 15 steps). To generate the ratiometric Raman images, the predicted scores for each spectrum of a Raman scan were calculated based on the DCLS model, after applying the same preprocessing as with the reference spectra. The scores on the reference spectrum corresponding to the αFR -NPs were divided point-wise by the scores on the nt-NP reference.

We devised an *in vitro* dilution scheme to test the effectiveness of our methodology. In a 1536-well plate we introduced suspensions of both αFR -NPs and nt-NPs, in various concentrations and ratios from 300 pM to 0.3 pM. The right- and left-most columns contain pure populations of αFR -NPs and nt-NPs, respectively. Each of the remaining columns contains a different ratio of αFR -NPs to nt-NPs, spanning 5 orders of magnitude (100:1 to 1:100) in decades. The predicted scores for the two NP references, as obtained from the DCLS model, are shown in Figure 2B, in a logarithmic scale. Since both the references and the data were normalized, any quantitative information about the signal intensity was lost. The scores reflect qualitatively how similar each point is to the reference, with a score of 1 (0 on the displayed logarithmic scale) being a perfect match. The scores on the two components are depicted in red for αFR -NPs and blue for nt-NPs. The model correctly identifies ratios of the two NP populations down to concentrations of 3 fM:300 fM (Figure 2C, bottom row).

To obtain the ratiometric image, the two scores were divided pointwise, and the result is displayed in Supplementary Figure S3. To demonstrate the potential of this methodology we use a logarithmic diverging color scale, with red showing αFR -NP:nt-NP ratios from 1:1 to 1000:1 and blue for ratios between 1:1 and 0.001:1. For the case of 1:1 ratio we used black.

Although this convention displays the most information possible for our system, we found it to be confusing when applied to complex geometries, such as the mouse anatomy. Thus, we decided to modify the color scheme, to make it more amenable to quick visual interpretation. As such, we limited the range of our color bar, covering ratios from 100:1 to 0.01:1 (2 to -2 in logarithmic scale), and instead of black for 1:1, we used a dark blue (Figure 2C). The rationale for this choice is that blue indicates healthy, and red cancer.

By using this regression model, ratiometry, and this color scheme, a wealth of information is provided in a quick, standardized way.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Suchetan Pal for advice regarding optimization of nanoparticle synthesis, Hsiao-Ting Hsu for help with cell culture and cell line characterization, as well as the MSKCC Electron Microscopy and Molecular Cytology core facilities for technical support. The following funding sources (to M.F.K.) are acknowledged: NIH R01 EB017748 and K08 CA16396; M.F.K. is a Damon Runyon-Rachleff Innovator supported (in part) by the Damon Runyon Cancer Research Foundation (DRR-29-14); Pershing Square Sohn Prize by the Pershing Square Sohn Cancer Research Alliance; MSKCC Center for Molecular Imaging & Nanotechnology (CMINT) Grant; MSKCC Technology Development Grant; Mr. William H. and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research and The Experimental Therapeutics Center of Memorial Sloan Kettering Cancer Center. AO was supported by a research fellowship from the German Research Foundation (OS 501/1-2). Acknowledgements are also extended to the grant-funding support provided by the MSKCC NIH Core Grant (P30-CA008748).

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Figure 1. Nanoparticle characterization

(A) Schematic depiction of nanoparticle structure. The gold nanostar core is encapsulated in a silica shell containing either IR780 (red) or IR140 (blue) Raman reporter dye. NPs are then functionalized with either a Folate Receptor targeting antibody (α FR-Ab) for targeted NPs (α FR-NPs, red) or with PEG (polyethylene glycol) for non-targeted NPs (nt-NPs, blue). (B) TEM Images of both NPs at 150,000 $\times$ magnification. (C) Size distribution of NPs measured by DLS shows an increase in size after silication of gold nanostar cores (from 78 nm to 122 nm for α FR-NPs and to 141 nm for nt-NPs) and functionalization with either α FR-Ab or PEG (final size 164 nm for both α FR-NPs and nt-NPs). (D) Zeta potential presents a significant change after functionalization (-28 mV for α FR-NPs and -5 mV for nt-NPs). (E) The absorbance maximum changed insignificantly after functionalization (gold nanocores 620 nm; α FR-NPs 640 nm; nt-NPs 650 nm).

Figure 2. Calibration of SERRS-NP Ratiometry

(A) Spectra from targeted α FR-NPs (red) and non-targeted nt-NPs (blue) were used as references for DCLS analysis. (B) α FR-NPs and nt-NPs were mixed at different ratios in a 1536-well plate with decreasing concentrations from top to bottom (y-axis: concentration of NPs). The left and right columns contain dilution series of pure nt-NPs and α FR-NPs, respectively. The remaining columns contain mixtures of both NPs in ratios of α FR-NPs to nt-NPs ranging from 1:100 to 100:1 in decades. The labels on the x-axes indicate the fractions of the concentrations displayed on the y-axes. DCLS analysis captures the

concentrations of both nanoprobe individually. (C) Ratiometric map based on DCLS scores, red indicating more α FR-NPs than nt-NPs and blue indicating a higher or equal concentration of nt-NPs than α FR-NPs.

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Figure 3. Whole abdomen imaging

of representative control (left) and tumor bearing (right) mice. Bioluminescence (BLI) signal is shown in the top row. The DCLS maps of both targeted (2nd row) and non-targeted (3rd row) show a non-specific distribution of both probes throughout the peritoneal cavity. TAS3RS (4th row) shows no positive regions in the control (left) and a strong correlation to BLI in tumor bearing mice (right). Alternatively TAS3RS map can be visualized in a simplified manner for surgical guidance (bottom row), only showing regions with positive ratios in red. Reference standard solutions in eppendorf vials were placed on the imaged field of view, with (1) indicating the vial containing αFR-NPs and (2) the vial containing nt-NPs.

Figure 4. Confirmation of the accuracy of TAS3RS imaging with BLI and histology

(A) Example of a multi-organ specimen on glass slide (photograph) of the upper abdomen with preserved anatomical orientation. This representative specimen demonstrates a good correlation between BLI (B) and the corresponding TAS3RS map (C). Three H&E slides (D) with an inter-slice gap of 250 μm (depth in μm indicated on the right) were obtained and examined for tumor lesions. Lesions at the same anatomical location are labeled with the same numerical annotation in the TAS3RS map (C) and histology slides (D) (white and black numbered arrows). Arrows 1-3: metastatic implants between the lesser curvature of the stomach and the liver hilum. Arrow 4: Large infra-gastric implant in pancreatic tissue. One perisplenic lesion (yellow arrow) was detectable with BLI and Raman, but not captured by histology, probably due to the interslice gaps.

Figure 5. Confirmation of the accuracy of TAS3RS imaging with BLI and histology in two additional mice

Bioluminescence (BLI) (A) and TAS3RS map (B) of two additional mice with preserved anatomical orientation. Three H&E slides with an inter-slice gap of 250 μm were obtained and reviewed for tumor lesions (C). Lesions at the same anatomical location are labeled with the same numerical annotation in the TAS3RS map and histology slides (white and black numbered arrows). Specimen of the upper abdomen (left column) with metastatic implants between the lesser curvature of the stomach and the liver hilum (arrows 1, 2). Large infra-gastric implant in pancreatic tissue (arrow 3). Lesions at cardia and greater curvature detectable with BLI and TAS3RS, but not captured in histologic correlation, probably due to

the interslice gap (yellow arrows). Pelvis (right column) with preserved urogenital organs and multiple peritoneal implants (arrows 1-5, 7, 8). One BLI and TAS3RS positive lesion was missed by histologic evaluation (yellow arrow). The ovarian lesion (arrow 6) is only partially captured by TAS3RS as it is covered by the right uterine horn, which correlates to the decreased and diffuse BLI signal.

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