A brain tumor molecular imaging strategy using a new triple-modality MRI-photoacoustic-Raman nanoparticle

Moritz F Kircher1–4,15, Adam de la Zerda1,5,15, Jesse V Jokerst4, Cristina L Zavaleta1, Paul J Kempen6, Erik Mittra1, Ken Pitter4,7, Ruimin Huang2,4,8, Carl Campos9, Frezghi Habte1, Robert Sinclair6, Cameron W Brennan4,9–11, Ingo K Mellinghoff8,9,12, Eric C Holland4,7,8,10,11,13 & Sanjiv S Gambhir1,6,14

The difficulty in delineating brain tumor margins is a major obstacle in the path toward better outcomes for patients with brain tumors. Current imaging methods are often limited by inadequate sensitivity, specificity and spatial resolution. Here we show that a unique triple-modality magnetic resonance imaging–photoacoustic imaging–Raman imaging nanoparticle (termed here MPR nanoparticle) can accurately help delineate the margins of brain tumors in living mice both preoperatively and intraoperatively. The MPRs were detected by all three modalities with at least a picomolar sensitivity both in vitro and in living mice. Intravenous injection of MPRs into glioblastoma-bearing mice led to MPR accumulation and retention by the tumors, with no MPR accumulation in the surrounding healthy tissue, allowing for a noninvasive tumor delineation using all three modalities through the intact skull. Raman imaging allowed for guidance of intraoperative tumor resection, and a histological correlation validated that Raman imaging was accurately delineating the brain tumor margins. This new triple-modality–nanoparticle approach has promise for enabling more accurate brain tumor imaging and resection.

The completeness of the surgical resection is a key factor in the prognosis of patients with brain tumors1,2. In attempting to achieve more complete glioma resections, the surgeon encounters several hurdles, including irregular and indistinct tumor margins, as well as tumor growth adjacent to or invading crucial neurological structures3. A wide variety of techniques have been explored in an effort to better visualize tumor margins. For example, preoperative magnetic resonance imaging (MRI) has been used to guide stereotactic surgery, where the magnetic resonance images are used to determine the macroscopic outline of the tumor4. However, such methods suffer from limited spatial resolution and incongruencies between the borders outlined in the preoperative MRI and the actual tumor borders during surgery because of brain shift5. Intraoperative MRI usually requires the administration of gadolinium (Gd) chelates, which have a short half-life and therefore require repeated injections6 and high dosages7 and the use of which may result in inaccuracies because of surgically induced false-positive contrast enhancement8. Several intraoperative optical methods have been suggested that are based either on intrinsic tissue optical properties9–11 or exogenous contrast agents10–12. However, these optical techniques have poor specificity because of tissue autofluorescence, limited resolution and depth of penetration, which ultimately limit the localization of the true brain tumor margins13,14.

Photoacoustic imaging is a new technology that largely overcomes the depth and resolution limits of optical imaging. In photoacoustic imaging, light pulses excite target molecular imaging agents, causing very slight heat production and thermal expansion. This produces ultrasound waves that are recorded by an ultrasound transducer that produces a three-dimensional image of the imaging agent’s distribution in living subjects15,16. Raman imaging, another promising and complementary optical imaging technique, can be greatly enhanced by the surface-enhanced Raman scattering (SERS) effect17. Because of the unique signature of the SERS spectrum, Raman imaging allows for highly specific and sensitive detection of SERS contrast agents, as well as the multiplexing of multiple agents in living subjects18–20.

An ideal molecular imaging agent would be sequestered and retained by a tumor for a long enough period that a single injection...
Figure 1 Triple-modality MPR concept. MPRs are injected intravenously into a mouse bearing an orthotopic brain tumor (top). As the nanoparticles circulate in the bloodstream, they diffuse through the disrupted blood-brain barrier and are then sequestered and retained by the tumor. The MPRs are too large to cross the intact blood-brain barrier and, therefore, cannot accumulate in healthy brain. The concept of proposed eventual clinical use (bottom). Detectability of MPRs by MRI allows for preoperative detection and surgical planning. Because of the retention of the probe, only one injection is necessary, and the probe can be detected in the tumor during surgery several days later. Photoacoustic imaging, with its relatively high resolution and deep tissue penetration, is then able to guide bulk tumor resection intraoperatively. Raman imaging, with its ultrahigh sensitivity and spatial resolution, can then be used to remove any residual microscopic tumor burden. The resected specimen can subsequently be examined using a Raman probe ex vivo to verify clear tumor margins.

of the agent would facilitate both preoperative and intraoperative imaging, allowing for preoperative planning and intraoperative resection of the tumor. This agent should also allow for both deep tumor visualization and highly sensitive and specific detection of tumor margins.

Here we present a new approach that attempts to fulfill these criteria. We have designed and tested MPRs for a triple-modality strategy that, to our knowledge, is the first to combine MRI, photoacoustic imaging and Raman imaging. This strategy achieves (i) whole-brain tumor localization for preoperative and intraoperative macroscopic delineation using MRI, (ii) high spatial resolution and three-dimensional localization for preoperative detection and surgical planning. Because of the retention of the maleimide-DOTA-Gd to the particles (Supplementary Methods), further modified our custom-made photoacoustic imaging system to include a 532-nm laser to allow for imaging of the MPRs (Supplementary Methods). The MPRs had a unique Raman signature (Fig. 2c), which was identical before and after the surface conjugation of the maleimide-DOTA-Gd to the particles (Supplementary Fig. 3).

RESULTS
Synthesis and characterization of triple-modality MPRs
The MPR nanoparticle is composed of a 60-nm gold core covered with the Raman molecular tag trans-1,2-bis(4-pyridyl)-ethylene. The thin Raman-active outer layer is protected by a 30-nm silica coating. We further modified the particles with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-Gd³⁺ using a maleimide linkage (maleimide-DOTA-Gd) (Online Methods), resulting in a gold-silica–based SERS nanoparticle coated with Gd³⁺ ions (the MPR nanoparticle) (Fig. 2a,b). We determined the number of Gd³⁺ ions per MPR to be 79,340 ± 2,270 (mean ± s.d.) using inductively coupled plasma atomic emission spectroscopy (ICP-AES). A portion of these Gd³⁺ ions could be bound directly to the silica surface. To test the serum stability of the MPRs, we performed ICP-AES and found that the number of Gd³⁺ ions per MPR decreased by only approximately 14% in the presence of mouse serum after 24 h of incubation (data not shown).

In addition, we validated that the MPRs are stable in serum by measuring their optical stability (Supplementary Fig. 1) and hydrodynamic size distribution (Supplementary Fig. 2) over the course of a 24-h incubation in mouse serum. We verified the stable binding of Gd³⁺ ions to the nanoparticle surface and the absence of any free Gd³⁺ ions in the solution by acquiring T1-weighted magnetic resonance images of the supernatant after particle centrifugation (data not shown). The MPRs showed a very high T1 relaxivity, 3.0 × 10⁶ M⁻¹ s⁻¹ in H₂O at a field strength of 7 T and at 20 °C, with minimal batch-to-batch variation (Fig. 2c). The MPR optical absorbance peaked at 540 nm and had a very high absorbance coefficient, 2.75 × 10¹⁰ cm⁻¹ M⁻¹ (Fig. 2d). We therefore further modified our custom-made photoacoustic imaging system to include a 532-nm laser to allow for imaging of the MPRs (Supplementary Methods). The MPRs had a unique Raman signature (Fig. 2c), which was identical before and after the surface conjugation of the maleimide-DOTA-Gd to the particles (Supplementary Fig. 3).
To test for possible photobleaching, we irradiated the MPRs using both the photoacoustic and the Raman imaging systems. During 30 min of continuous laser irradiation, the optical absorption and Raman signal each did not vary more than 2% (Fig. 2f,g and Supplementary Methods).

**In vitro and in vivo detection of MPRs**

We next determined the in vitro detection threshold of the MPRs for each modality. We imaged an agarose phantom containing MPRs in concentrations ranging from 1.22 pM to 1.250 pM (n = 3 per concentration) using MRI, photoacoustic imaging and Raman imaging (Supplementary Fig. 4b) and determined the signal intensities using a region-of-interest analysis (Supplementary Fig. 4b). The lowest detectable concentrations were 4.88 pM for MRI, 1.22 pM for photoacoustic imaging and 610 fM for Raman imaging (Supplementary Fig. 5). The MRI, photoacoustic and Raman signals produced by the MPRs in vitro were highly correlated with the MPR concentrations (P < 0.0001 for all modalities, with $R^2 = 0.97$, 0.99 and 0.99 for MRI, photoacoustic and Raman, respectively) (Supplementary Fig. 4b) and were also highly linear and correlated with each other (Supplementary Fig. 6). For a comparison of the depth of penetration of photoacoustic imaging compared to Raman imaging, see Supplementary Figure 7.

We then measured the detection thresholds of the MPRs in living mice. We subcutaneously injected MPRs diluted in matrigel to six different concentrations (ranging from 50 pM to 1,100 pM) into the right flanks of nude mice (n = 3 mice) and scanned the mice using the MRI, photoacoustic and Raman systems (Supplementary Fig. 8a). The MRI, photoacoustic and Raman signals in vivo highly correlated to the MPR concentrations (P = 0.001 for all modalities, with $R^2 = 0.99, 0.97$ and 0.99 for MRI, photoacoustic and Raman, respectively) (Supplementary Fig. 8b). The sensitivity of the MRI and the photoacoustic imaging was limited by the intrinsic tissue background signal. For both MRI and photoacoustic imaging, 50 pM of MPRs gave an equivalent signal to muscle. Raman imaging, however, had a negligible tissue background signal and was therefore limited only by the signal-to-noise ratio. Indeed, at the nominal concentration of 50 pM, the Raman image clearly visualized the MPRs. This explains why the Raman response had the steepest slope in vivo as compared to those from the MRI and photoacoustic imaging (P < 0.0001 for Raman imaging compared to either MRI or photoacoustic imaging; however, the slope of the photoacoustic imaging was not statistically different from that of MRI, $P = 0.16$), whereas in vitro, where no substantial background signal was present, photoacoustic imaging had the steepest slope of the three modalities. Finally, we found a linear correlation between the signals of the three modalities (Supplementary Fig. 9).

**In vivo triple-modality brain tumor visualization of MPRs**

We next sought to determine whether the MPRs could be used for orthotopic brain tumor detection in living mice. We hypothesized that in an orthotopic glioblastoma brain tumor model, the nanoparticle probe would enter the extravascular space by diffusion through the disrupted blood-brain barrier and accumulate in cells within the tumor without necessitating a specific targeting mechanism (enhanced permeability and retention (EPR) effect), as has been previously observed for iron oxide nanoparticles. We used an orthotopic brain tumor model in which we implanted enhanced GFP (eGFP)-transfected human glioblastoma cells (eGFP*U87MG) into the striata of nude mice using a stereotactic implantation device (Supplementary Methods). We injected tumor-bearing mice (n = 4) through tail vein with MPRs and performed consecutive photoacoustic imaging, Raman imaging and MRI on each mouse before injection and at 2 h, 3 h and 4 h after injection, respectively (Fig. 3 and Supplementary Fig. 10).

The images from after the injection demonstrated clear visualization of the tumor for all three modalities despite having been acquired through the intact skin and skulls of the mice (Fig. 3a). The photoacoustic and Raman images were co-registered with the MRI image, showing good colocalization between the three modalities (Fig. 3a). In parallel to the photoacoustic images of the brain, we also acquired co-registered ultrasound images to register the photoacoustic images to the magnetic resonance images in orthogonal planes (Fig. 3b) (using Amide and Amira software, see Supplementary Methods).

A region of interest quantification of the signals in the tumors showed a significant increase in the MRI, photoacoustic and Raman signals after as compared to before the tail-vein injection (Fig. 3c). The MRI contrast-to-noise ratio increased from 2.2 ± 0.3 to 14.0 ± 1.9.

**Figure 3** Triple-modality detection of brain tumors in living mice with MPRs. (a) Two-dimensional axial MRI, photoacoustic and Raman images. The post-injection images of all three modalities showed clear tumor visualization (dashed boxes outline the imaged area). (b) A three-dimensional (3D) rendering of magnetic resonance images with the tumor segmented (red; top), an overlay of the three-dimensional photoacoustic images (green) over the MRI (middle) and an overlay of the three-dimensional photoacoustic and Raman images (bottom) showing good colocalization of the photoacoustic signal with the tumor. (c) Quantification of the signals in the tumor showing a significant increase in the MRI, photoacoustic and Raman signals after as compared to before the injection. n = 4 mice. Data represent mean ± s.e.m. **P < 0.001, *P < 0.01** (one-sided Student’s *t* test.) AU, arbitrary units.
Figure 4 Histological validation. Ten-micrometer frozen sections from the margin of an eGFP*U87MG brain tumor stained for eGFP (green) to visualize the tumor margins and CD11b (red) to visualize glial cells and examined using laser scanning confocal microscopy (top). A 50-µm adjacent slice examined using Raman microscopy to visualize the distribution of the MPRs (bottom). The Raman signal corresponding to the eGFP* cells indicates the presence of the probe in the tumor but not in the adjacent healthy tissue. The Raman signal (red) was scaled from 0 to 100 AU. The boxes are not drawn to scale. STEM images verify the presence of MPRs in the brain tissue, whereas no MPRs were seen in the healthy brain tissue. A three-dimensional STEM rendering of MPRs in brain tumor is provided in Supplementary Video 1.

(mean ± s.e.) (P = 0.001). The photoacoustic signal increased by 75%, from 0.57 ± 0.02 AU to 1.0 ± 0.08 AU (P = 0.001). The Raman system recorded no signal before injection and an intense signal of 1.0 ± 0.09 AU (P = 0.012) after injection.

We then determined the kinetics of the MPRs for all three modalities using additional mice orthotopically implanted with eGFP*U87MG tumor cells (n = 4 mice each for MRI and Raman imaging; we derived the photoacoustic data from the first set of mice described for Fig. 3). We analyzed the signal kinetics for each mouse individually for the MRI, photoacoustic and Raman imaging systems (Supplementary Fig. 11). We acquired data before injection and at 0.5 h, 1 h, 1.5 h and 2 h after injection. For MRI, we also acquired data 24 h after injection. We found that the MPR signal increased markedly between the pre-injection and the 30-min post-injection time points for all three modalities (from 1.4 ± 0.24 to 8.7 ± 0.76 contrast-to-noise ratio for MRI (P < 0.001), a 60% ± 14% increase for photoacoustic imaging (P < 0.01) and from 0 to 1.96 ± 0.27 AU for Raman imaging (P < 0.001)). The signal then reached a plateau for MRI and photoacoustic imaging, which remained essentially stable until the latest examined time point of 2 h for photoacoustic imaging and 24 h for MRI. Of note, this behavior contrasts with conventional clinically used Gd-based contrast agents, which show rapid washout within minutes after injection, whereas the MPRs showed persistent signal enhancement (Supplementary Fig. 11). For Raman imaging, we observed an initial signal peak before the signal reached a plateau (P < 0.0001 for the peak value compared to the plateau). We presumed this effect was caused by the initial nonspecific circulation of MPRs in superficial layers (for example, skin), to which Raman imaging is most sensitive of the three modalities (Supplementary Fig. 11).

Histological validation of MPR sequestration by brain tumors
We next examined the distribution of the MPRs within the brain using histology. Using the mice described above, we performed immunohistochemistry with antibody staining against eGFP and CD11b to visualize eGFP*U87MG tumor cells and microglia, respectively. In particular, we sampled sections that included the interface between the tumor and the surrounding brain tissue. We then examined adjacent sections using high-resolution Raman microscopy (Supplementary Methods) and correlated these images with the immunohistochemistry results. We observed a strong Raman signal within the tumors but not in healthy brain tissue, with the Raman signal producing a very good delineation of the actual tumor border (Fig. 4). Scanning transmission electron microscopy (STEM) (Fig. 4) further corroborated these results, showing numerous MPRs in tumor sections and none in the surrounding brain tissue (923 MPRs found within tumors in an examined volume of 57,500 μm³ (average of 0.016 MPRs per μm³) and no MPRs found in healthy brain in an examined volume of 12,500 μm³). A three-dimensional STEM rendering of MPRs in the tumors is provided in Supplementary Video 1.

To further examine the ability of the MPRs to visualize not only the bulk tumor but also the margins of the invasive tumor, we used an orthotopic primary human xenograft glioblastoma mouse model (the TS543 cell line grown as neurospheres). As confirmed by correlative Raman microscopy, immunohistochemistry and scanning electron microscopy, the MPRs accumulated in the infiltrating tumor margins (Supplementary Fig. 12). In addition, Raman imaging was able to depict finger-like tumor protrusions and even isolated microscopic tumor foci (Supplementary Fig. 13).

MPRs guide brain tumor resection in vivo
Finally, we explored whether tumor resection along the photoacoustic and Raman signals, 24 h after intravenous injection of MPRs, could facilitate tumor resection. Initially, we tested the ability of

Figure 5 Raman-guided intraoperative surgery using MPRs. (a,b) Living tumor-bearing mice (n = 3) underwent craniotomy under general anesthesia. Quarters of the tumor were then sequentially removed (as illustrated in the photographs, a), and intraoperative Raman imaging was performed after each resection step (b) until the entire tumor had been removed, as assessed by visual inspection. After the gross removal of the tumor, several small foci of Raman signal were found in the resection bed (outlined by the dashed white square; some Raman images are smaller than the image frame). The Raman color scale is shown in red from −40 dB to 0 dB. (c) A subsequent histological analysis of sections from these foci showed an infiltrative pattern of the tumor in this location, forming finger-like protrusions extending into the surrounding brain tissue. As shown in the Raman microscopy image (right), a Raman signal was observed within these protrusions, indicating the selective presence of MPRs. The box is not drawn to scale. The Raman signal is shown in a linear red color scale.
photoacoustic imaging to delineate brain tumors in situ, which showed a reduced signal in the resected area (Supplementary Fig. 14). Next, we placed brain-tumor-bearing mice (n = 3) under general anesthesia and performed craniotomies and subsequent in vivo Raman imaging. We then removed sections of the brain tumors detected using visual inspection only. We obtained high-resolution intraoperative Raman images after each resection step and correlated them with the intraoperative photographs. We first visualized the whole tumor using Raman imaging (Fig. 5a,b). With each sequential resection step, we noted a high congruency between residual tumor tissue (Fig. 5a) and the presence of a Raman signal (Fig. 5b) and, vice versa, between resected tumor and lack of a Raman signal. Of note, after the tumor resection seemed to be complete by visual inspection, we noted several small foci of residual Raman signal (Fig. 5b) in the resection bed. When we then extended the resection to include these foci, which were located near the tumor-brain interface and histologically analyzed this tissue, we found frequent finger-like microscopic extensions of the tumor into the surrounding brain tissue (Fig. 5c). We were able to detect these cancerous foci, which were otherwise not visible to the naked eye because of the specific accumulation of the MPRs therein.

**DISCUSSION**

We designed and tested a unique triple-modality nanoparticle that is, to our knowledge, the first to combine MRI, photoacoustic and Raman imaging. The MPRs described here could enable radiologists and neurosurgeons to ‘see’ the same probe before and during surgery, thus allowing for more accurate brain tumor resection by exploiting the complementary strengths of each modality.

The excellent detectability of MPRs using MRI (in the picomolar range) is a direct result of the very high longitudinal relaxivity of the MPRs of $3.0 \times 10^6 \, \text{mM}^{-1} \, \text{s}^{-1}$. To our knowledge, this is the highest relaxivity of a nanoparticle reported to date.

Photoacoustic imaging is a relatively new technique that allows deeper tissues to be imaged with higher spatial resolution compared to most optical techniques$^{25-27}$. The exceptionally high optical absorbance coefficient of the MPRs is over 200-fold higher than, for example, previously reported photoacoustic imaging agents that are based on carbon nanotubes$^{28,29}$. Also considering its three-dimensional capabilities, photoacoustic imaging could guide the more gross resection steps and even identify tumor tissue residing under the surface of normal brain tissue. Then, to completely remove microscopic tumor deposits, Raman imaging, with its superior sensitivity, could be used.

Raman imaging in conjunction with MPRs offers ultrahigh sensitivity in the picomolar range, as opposed to the nanomolar sensitivity that is achievable using fluorescence imaging of quantum dots$^{13,17,18,20}$. Raman imaging of MPRs, in contrast to other optical imaging techniques, does not suffer from autofluorescence or background signal because the MPR spectral signature is highly amplified and unique (its ‘fingerprint’). Although the main limitation of Raman imaging is its limited penetration depth, we achieved tumor visualization here through the intact skin and skulls of live mice (to a depth of 2–5 mm). This result stems from a combination of the design of the nanoparticle, with its gold core producing a surface plasmon resonance for Raman signal enhancement, the Raman substrate used and the number of nanoparticles accumulating within the tumor. Raman nanoparticles are inherently insensitive to photodestruction, which is a known problem of organic fluorochromes. Furthermore, unlike most quantum dots, which are cytotoxic$^{30,31}$, MPR nanoparticles are based on inert gold and silica and, thus, may have a better chance for clinical translation. Gold and gold-silica nanoparticles have excellent cytotoxicity profiles, as has been illustrated in detailed toxicity studies in cell cultures, mice$^{32-34}$ and several clinical trials$^{21}$. The design of the MPRs would also allow for multiplexing$^{35}$, with the potential to detect multiple biomarkers simultaneously in vivo.

In addition, MPRs have a unique advantage over conventional contrast agents of low molecular weight. For example, low–molecular-weight Gd chelates or fluorochromes accumulate in the extracellular space, where breakdown of the blood–brain barrier has occurred, and then undergo rapid diffusion through the interstitium and renal clearance. These low–molecular-weight agents are therefore unable to delineate tumors for the time period spanning the resection procedure, let alone for the entire period between preoperative planning and surgical intervention. This diffusion process also introduces imprecision of probe localization, requires repeated contrast administration (for example, of Gd chelates during intraoperative MRI) and can cause false-positive results because of surgically induced contrast enhancement. In contrast, the in vivo kinetic studies performed with the MPRs here show that the particle is retained in the tumor, allowing for repeated imaging as required without the need for multiple injections. This contrast-agent behavior may also be useful for distinguishing tumor recurrence from nonspecific treatment-related effects. As the MPR approach relies on the EPR effect, it could potentially be applied to imaging other cancer types that have intrinsic EPR effects, including lung cancer, melanoma, renal cancer, hepatoma and many others$^{35}$. In addition, the long intratumoral retention of the MPRs could also be exploited for drug delivery or photothermal therapy.

New instrumentation, including endoscopic and intraoperative photoacoustic and Raman imaging devices that are required for the clinical translation of the MPR approach described here, are currently under development$^{36,37}$. Ideally, a combination of both devices integrated into one handheld probe would be available in the operating room. In particular, such endoscopes should be designed for easy intraoperative navigation and should enable real-time imaging. Further development of such instrumentation could lead to improved brain tumor surgery and outcomes for patients in the future. For an additional discussion, see the Supplementary Discussion.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

**ACKNOWLEDGMENTS**

We thank M. Gozin for help with ICP-AES, L. Pisani for assistance with quantifying and acquiring magnetic resonance data, S.M. Korn and S. Bodapati for assistance in conducting the photoacoustic experiments, J. Rosenberg for assistance with the statistical analysis and the Memorial Sloan-Kettering Cancer Center Animal Imaging Core Facility (J. Koutcher and C.C. Le) for technical assistance. M.F.K. would like to thank R. Herfkens and the Stanford Department of Radiology for providing academic time to perform the study. We would like to acknowledge the following funding sources: National Cancer Institute grants CCNE U54 CA119367 (S.S.G.), CCNE U54 U54CA151459 (S.S.G.) and ICMIC P50 CA114747 (S.S.G.); The Ben and Catherine Ivy Foundation (S.S.G.); the Canary Foundation (S.S.G.); the Sir Peter Michael Foundation (S.S.G.); the Bio-X Graduate Student Fellowship (A.D.L.Z.); the Department of Defense Breast Cancer Research Program—Pre-doctoral Traineeship Award BC083014 (A.d.l.Z.) and the National Cancer Institute SMIS R2ST Fellowship 5R25CA118681 (J.V.J.).

The authors would also like to thank T.F. Massoud, D. Akin, H.E. Daldrop-Link, S. Bohndiek, S. Harmesen and J. Samit for critical review of the manuscript and B.T. Khuri-Yakub, S. Vaithilingam, O. Oralkan, E.E. Graves and H. Fan-Minogue for helpful discussions.
TECHNICAL REPORTS

AUTHOR CONTRIBUTIONS
M.F.K. co-initiated the project, designed the research, synthesized and characterized MPR nanoparticles, performed MRI, Raman, photoacoustic and histology experiments, analyzed data and wrote the manuscript. A.d.I.Z. modified the photoacoustic system, designed and performed photoacoustic experiments, analyzed data and wrote the manuscript. J.V.J. synthesized and characterized MPR nanoparticles. C.I.Z. designed, performed and analyzed Raman experiments and edited the paper. P.I.K. and R.S. performed and analyzed the electron microscopy experiments. K.P. performed immunohistochemistry. E.H. helped create three-dimensional renderings. E.M., M.F.K., R.H., C.C., C.W.B., I.K.M. and E.C.H. provided mouse models. S.S.G. co-initiated the project, designed the research, supervised and coordinated all investigators for the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Bucci, M.K. et al. Near complete surgical resection predicts a favorable outcome in pediatric patients with nonbrainstem, malignant gliomas: results from a single center in the magnetic resonance imaging era. Cancer 101, 817–824 (2004).
2. Stupp, R. et al. Changing paradigms—an update on the multidisciplinary management of malignant glioma. Oncologist 11, 165–180 (2006).
3. Toms, S.A. et al. Intraoperative optical spectroscopy identifies infiltrating glioma margins with high sensitivity. Neurosurgery 57, 382–391, discussion 382–391 (2005).
4. Orringer, D.A. et al. The brain tumor window model: a combined cranial window and implanted glioma model for evaluating intraoperative contrast agents. Neurosurgery 66, 736–743 (2010).
5. Reinges, M.H. et al. Course of brain shift during microsurgical resection of supratentorial cerebral lesions: limits of conventional neuronavigation. Acta Neurochir. (Wien) 146, 369–377, discussion 377 (2004).
6. Lüdemann, L., Hamm, B. & Zimmer, C. Pharmacokinetic analysis of glioma compartments with dynamic Gd-DTPA-enhanced magnetic resonance imaging. Magn. Reson. Imaging 18, 1201–1214 (2000).
7. Knauth, M., Wirtz, C.R., Aras, N. & Sartor, K. Low-field interventional MRI in neurosurgery: finding the right dose of contrast medium. Neuroradiology 43, 254–258 (2001).
8. Knauth, M. et al. Surgically induced intracranial contrast enhancement: potential source of diagnostic error in intraoperative MR imaging. AJNR Am. J. Neuroradiol. 20, 1547–1553 (1999).
9. Bejebbar, A., Dukic, S., Amharref, N. & Manfait, M. Ex vivo and in vivo diagnosis of C6 glioblastoma development by Raman spectroscopy coupled to a microprobe. Anal. Bioanal. Chem. 398, 477–487 (2010).
10. Ozawa, T. et al. Bromophenol blue staining of tumors in a rat glioma model. Neurosurgery 57, 1041–1047, discussion 1041–1047 (2005).
11. Shindoh, J. et al. Fluorescence-guided resection of glioblastoma multiforme by using high-dose fluorescein sodium. Technical note. J. Neurosurg. 99, 597–603 (2003).
12. Stummer, W. et al. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. Lancet Oncol. 7, 392–401 (2006).
13. de la Zerda, A. et al. A comparison between time domain and spectral imaging systems for imaging quantum dots in small living animals. Mol. Imaging Biol. 12, 500–508 (2010).
14. Kanelhhardt, S.R. et al. Multiphoton excitation fluorescence microscopy of 5-aminolevulinic acid induced fluorescence in experimental gliomas. Lasers Surg. Med. 40, 273–281 (2008).
15. de la Zerda, A. et al. Carbon nanotubes as photoacoustic molecular imaging agents in living mice. Nat. Nanotechnol. 3, 557–562 (2008).
16. Wang, L.V. Multiscale photoacoustic microscopy and computed tomography. Nat. Photonics 3, 503–509 (2009).
17. Zavaleta, C.L., Kircher, M.F. & Gambhir, S.S. Raman’s “effect” on molecular imaging. J. Nucl. Med. 52, 1839–1844 (2011).
18. Keren, S. et al. Noninvasive molecular imaging of small living subjects using Raman spectroscopy. Proc. Natl. Acad. Sci. USA 105, 5844–5849 (2008).
19. Zavaleta, C.L. et al. Noninvasive Raman spectroscopy in living mice for evaluation of tumor targeting with carbon nanotubes. Nano Lett. 8, 2800–2805 (2008).
20. Zavaleta, C.L. et al. Multiplexed imaging of surface enhanced Raman scattering nanotags in living mice using noninvasive Raman spectroscopy. Proc. Natl. Acad. Sci. USA 106, 13511–13516 (2009).
21. Adishehia, P.P., Hall, J.B. & McNeil, S.E. Nanomaterial standards for efficacy and toxicity assessment. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 2, 99–112 (2010).
22. Trehin, R. et al. Fluorescent nanoparticle uptake for brain tumor visualization. Neoplasia 8, 302–311 (2006).
23. Loening, A.M. & Gambhir, S.S. AMIDE: a free software tool for multimodality medical image analysis. Mol. imaging 2, 131–137 (2003).
24. Vivanco, I. et al. The phosphatase and tensin homolog regulates epidermal growth factor receptor (EGFR) inhibitor response by targeting EGFR for degradation. Proc. Natl. Acad. Sci. USA 107, 6459–6464 (2010).
25. Ermilov, S.A. et al. Laser optoacoustic imaging system for detection of breast cancer. J. Biomed. Opt. 14, 024007 (2009).
26. Manohar, S. et al. Initial results of in vivo non-invasive cancer imaging in the human breast using near-infrared photoacoustics. Opt. Express 15, 12277–12285 (2007).
27. de la Zerda, A. et al. Photoacoustic ocular imaging. Opt. Lett. 35, 270–272 (2010).
28. de la Zerda, A. et al. Ultrahigh sensitivity carbon nanotube agents for photoacoustic molecular imaging in living mice. Nano Lett. 10, 2168–2172 (2010).
29. de la Zerda, A., Kim, J.W., Galanlaza, E.I., Gambhir, S.S. & Zharov, V.P. Advanced contrast nanogens for photoacoustic molecular imaging, cytometry, blood test and photothermal theranostics. Contrast Media Mol. Imaging 6, 346–369 (2011).
30. Ziu, S. et al. Weight of evidence approach for the relative hazard ranking of nanomaterials. Nanotoxicology 5, 445–458 (2011).
31. Kircher, M.F., Gambhir, S.S. & Grimm, J. Noninvasive cell-tracking methods. Nat. Rev. Clin. Oncol. 8, 677–688 (2011).
32. Thakor, A.S. et al. The fate and toxicity of Raman-active silica-gold nanoparticles in mice. Sci. Transl. Med. 3, 79ra33 (2011).
33. Thakor, A.S. et al. Oxidative stress mediates the effects of Raman-active gold nanoparticles in human cells. Small 7, 126–136 (2011).
34. Zavaleta, C.L. et al. Preclinical evaluation of Raman nanoparticle biodistribution for their potential use in clinical endoscopy imaging. Small 7, 2232–2240 (2011).
35. Maeda, H., Wu, J., Sawa, T., Matsumura, Y. & Hori, K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Control. Release 65, 271–284 (2000).
36. Koljenovic, S. et al. Raman spectroscopic characterization of porcine brain tissue using a single fiber-optic probe. Anal. Chem. 79, 557–564 (2007).
37. Short, M.A. et al. Development and preliminary results of an endoscopic Raman probe for potential in vivo diagnosis of lung cancers. Opt. Lett. 33, 711–713 (2008).
ONLINE METHODS

Particle synthesis. Reagents. SERS nanotags (Cabot Security Materials, Inc.) comprised a 60-nm diameter gold core that was coated with a monolayer of a Raman-active organic molecule, trans-1,2-bis(4-pyridyl)-ethylene, and was encapsulated with a 30-nm–diameter silica shell, making the entire particle on the order of ~120 nm. We purchased PBS, 2-(N-morpholino)ethanesulfonic acid (MES), 3-mercaptopropyl-trimethoxysilane (MPTMS), gadolinium chloride hexahydrate and sodium chloride from Sigma-Aldrich, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman’s reagent) from Pierce and 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidodeethylacetamide (maleimide-DOTA) from Macrocyclics.

Bioconjugation. We activated the SERS nanotag surface with 10 mM MES buffer, pH 7.0, for binding of maleimide-DOTA to the mercaptoalkyltrimethoxysilane (MPTMS)-coated surface. We added the DOTA chelator at a molar excess of 7.5 × 10^5 per nanoparticle from a stock solution of 1 mg/ml, reacted it for 2 h at room temperature and removed the excess reagent using centrifugation (5,000 g for 5 min) three times. We added gadolinium chloride hexahydrate (3 mg/ml) to the DOTA-activated nanoparticles in MES buffer (pH 6.25), with a ratio of Gd to nanoparticle of 7.5 × 10^5:1. We heated this solution to 50 °C for 2 h, washed it three times, as above, and then purified it by dialysis versus distilled water and a 3.5-kDa molecular weight cutoff membrane. The optical absorbance was measured using an ultraviolet-visible (UV-Vis) spectrophotometer (DU-640 spectrophotometer, Beckman Coulter).

MRI. We conducted MRI scans with a dedicated small-animal MRI scanner, custom-designed pulse sequences and radiofrequency coils. The small-animal scanner consisted of a superconducting magnet (Magnex Scientific) with 7.0 T field strength, a gradient (Resonance Research, Inc.) with a clear bore size of 7 cm, and an MRI console and Copley 266 amplifiers. The scanner consisted of a superconducting magnet (Magnex Scientific) with 7.0 T field strength, a gradient (Resonance Research, Inc.) with a clear bore size of 7 cm, and a General Electric console and Copley 266 amplifiers. We obtained T1-weighted fast-spin echo sequences (echo time (TE)/repetition time (TR) = 7.7/800 ms) using 5 NEX, a 256 × 256 matrix, a 3.0-cm field of view, and 4.2 F number, 7.5 mm depth of focus, 250 MHz focused transducer (V324-SU-25.5-MM; 27 mm focal length, 12 MHz bandwidth, 6.5 mm depth of focus, 600 µm lateral resolution and 380 µm axial resolution; Panametrics-Olympus NDT) to acquire both pulse-echo and photoacoustic images. In addition, we acquired high-resolution ultrasound images using a 5-MHz focused transducer (A309S-SU-F-24.5-MM-PTF; 25.5 mm focal length, 4 MHz bandwidth, 2.0 F number, 6.5 mm depth of focus, 600 µm lateral resolution and 380 µm axial resolution; Panametrics-Olympus NDT) to acquire pulse-echo and photoacoustic images. In addition, we acquired high-resolution ultrasound images using a 25-MHz focused transducer (V324-SU-25.5-MM; 27 mm focal length, 12 MHz bandwidth, 4.2 F number, 7.5 mm depth of focus, 250 µm lateral resolution and 124 µm axial resolution; Panametrics-Olympus NDT). We purchased PBS, 2-(N-morpholino)ethanesulfonic acid (MES), 3-mercaptopropyl-trimethoxysilane (MPTMS), gadolinium chloride hexahydrate and sodium chloride from Sigma-Aldrich, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman’s reagent) from Pierce and 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidodeethylacetamide (maleimide-DOTA) from Macrocyclics.

Additional methods. Detailed methodology is described in the Supplementary Methods.

38. Graves, E.E., Quon, A. & Loo, B.W. Jr. RT_Image: an open-source tool for investigating PET in radiation oncology. Technol. Cancer Res. Treat. 6, 111–121 (2007).
39. American National Standards Institute. American national standard for the safe use of lasers. in ANSI Standard Z136.1–2000 (ANSI, Inc., New York, 2000).