Published in final edited form as:

Nat Nanotechnol. 2009 December ; 4(12): 855–860. doi:10.1038/nnano.2009.333.

In vivo magnetic enrichment and multiplex photoacoustic detection of circulating tumour cells

Ekaterina I. Galanzha^{1,2}, Evgeny V. Shashkov^{1,3}, Thomas Kelly^{1,4}, Jin-Woo Kim⁵, Lily Yang⁶, and Vladimir P. Zharov^{1,*}

¹Phillips Classic Laser and Nanomedicine Laboratories, Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205, USA

²Saratov State University, Institute of Optics and Biophotonics, Saratov 410012, Russia

³Prokhorov General Physics Institute, Moscow 119991, Russia

⁴Department of Pathology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205, USA

⁵Department of Biological and Agricultural Engineering and Institute for Nanoscale Materials Science and Engineering, University of Arkansas, Fayetteville, Arkansas 72701, USA

⁶Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia 30322, USA

Abstract

The spread of cancer cells between organs, a process known as metastasis, is the cause of most cancer deaths^{1,2}. Detecting circulating tumour cells—a common marker for the development of metastasis^{3,4}—is difficult because ex vivo methods are not sensitive enough owing to limited blood sample volume and in vivo diagnosis is time-consuming as large volumes of blood must be analysed^{5–7}. Here, we show a way to magnetically capture circulating tumour cells in the bloodstream of mice followed by rapid photoacoustic detection. Magnetic nanoparticles, which were functionalized to target a receptor commonly found in breast cancer cells, bound and captured circulating tumour cells under a magnet. To improve detection sensitivity and specificity, gold-plated carbon nanotubes conjugated with folic acid were used as a second contrast agent for photoacoustic imaging. By integrating in vivo multiplex targeting, magnetic enrichment, signal amplification and multicolour recognition, our approach allows circulating tumour cells to be concentrated from a large volume of blood in the vessels of tumour-bearing mice, and this could have potential for the early diagnosis of cancer and the prevention of metastasis in humans.

Immunomagnetic microbeads and magnetic nanoparticles (MNPs) have been extensively explored in magnetic resonance imaging, hyperthermia, targeted delivery of therapeutics and separation of cells^{8–17}. In this study, we developed a new platform for in vivo magnetic enrichment and detection of rare circulating tumour cells (CTCs) from a large pool of blood using targeted MNPs in combination with two-colour photoacoustic flow cytometry.

Correspondence and requests for materials should be addressed to V.P.Z. zharovvladimirp@uams.edu.

Author contributions

E.I.G. and V.P.Z. conceived and designed the experiments. J.-W.K. was responsible for GNT synthesis and their characterization and bioconjugation, L.Y. for conjugation and characterization of MNPs, T.K. for providing assessments of cancer cells and E.I.G. and E.V.S. for the remaining experiments. All authors discussed the results. V.P.Z., E.I.G. and J.-W.K. co-wrote the paper with editorial comments from L.Y. and T.K.

Additional information

Supplementary information accompanies this paper at www.nature.com/naturenanotechnology. Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>.

Although photoacoustic imaging has higher spatial resolution in deep tissues (up to 3 cm) than other optical modalities^{18,19}, the slow signal acquisition algorithms cannot detect fast-moving CTCs in the bloodstream. To solve this problem, we modified the previously described time-resolved photoacoustic technique^{20–22} so that CTCs targeted by two-colour nanoparticles can be illuminated by laser pulses at wavelengths of 639 and 900 nm at 10-ms delay (Fig. 1a; see Methods and Supplementary Fig. S1). Because human tumour cells are heterogeneous and not all tumour cells express a given biomarker²³, we applied multiplex targeting and a multicolour detection strategy^{21,24,25} to increase the specificity of the nanoparticles so that in vivo CTC identification was possible.

MNPs were conjugated to the amino-terminal fragment of the urokinase plasminogen activator, which serves as a ligand that binds specifically to the urokinase plasminogen activator receptors that are highly expressed on many types of cancer cells but are expressed at a low level in normal blood and endothelial cells^{26,27} (Fig. 1b). The MNPs served as dual magnetic and photoacoustic contrast agents using the intrinsic absorption of their Fe₂O₃ core (Supplementary Fig. S4). Estimated photoacoustic spectra of MNPs suggest that 639 nm is the preferred wavelength because it provides the best photoacoustic contrast in a background of blood (Fig. 1d). To increase sensitivity and specificity, golden carbon nanotubes (GNTs)²⁸ (Fig. 1c; see Supplementary Information), which have a higher photoacoustic contrast than MNPs at 900 nm (Fig. 1d), were used as a second molecular agent for targeting the folate receptors that are expressed in cancer cells but absent in normal blood⁷. The human breast cancer cell line MDA-MB-231, which is positive for both the urokinase plasminogen activator and the folate receptor, was used to establish a human breast cancer xenograft model in nude mice.

To verify the target specificity of the nanoparticles in vitro, mouse blood samples were spiked with MDA-MB-231 cells and treated with unconjugated and conjugated nanoparticles either alone or in a cocktail (GNT/MNP proportion of 20:80) under static or flow conditions. Targeting was confirmed by the presence of nanoparticles in cancer cells as shown by Prussian Blue staining (Fig. 2a), fluorescent imaging (Fig. 2b; Supplementary Fig. S5a) and magnetic resonance imaging (Supplementary Fig. S5b). Photoacoustic signals from MNP-labelled cells were significantly higher than MNPs alone, particularly at high laser fluence and after exposure to a magnet for 10 min (Fig. 2c). This signal amplification was associated with magnet-induced MNP clustering in cells and laser-induced microbubbles²² around the MNP clusters. Measurement of photoacoustic signals as a function of number of nanoparticles revealed that the sensitivity limit is 35 GNTs and 720 MNPs (Fig. 2d; see Supplementary Information). The nanoparticle cocktail showed the best targeting efficiency (96±2.1%) after ~1 h labelling under static conditions or after 5–10 min under flow conditions at a velocity of 0.1 cm s⁻¹ (Table 1).

Attaching a magnet with a field strength of 0.39 T to the testing tube (Fig. 3a) captured 10-nm MNP-labelled cells in phosphate buffer saline (PBS; Fig. 3b) at a broad range of flow velocity (0.1–10 cm s⁻¹), and this was accompanied by strong photoacoustic signals from the area under the magnet (Fig. 3e) that were stronger than those signals outside the magnet corresponding to rare uncaptured cells and unbound MNPs (Fig. 3f). The introduction of free MNPs at a high concentration (1 × 10¹¹ MNPs ml⁻¹) with a small number (5–10) of MNP-labelled cells demonstrated that both MNPs and MNP-labelled cells were captured at a flow velocity of 0.1 cm s⁻¹ (Fig. 3c). Increasing the velocity to 3 cm s⁻¹ removed most of the free MNPs but the MNP-labelled cell remained captured (Fig. 3d). Because magnetic force is proportional to particle number¹⁷, the randomly distributed free MNPs were more effectively removed by flow drag forces than the labelled cells that contained a higher local MNP concentration or dense MNP clusters. As a result, labelled cells were captured

effectively up to a flow velocity of between 3 and 5 cm s⁻¹, but free MNPs were captured only at a low velocity between 0.3 and 0.5 cm s⁻¹ (Fig. 3g).

To determine the depletion kinetics, MNPs and GNTs were separately injected, intravenously, through mouse tail vein at concentrations of 1×10^9 and 1×10^{11} nanoparticles ml⁻¹ in 10 ml PBS (n = 3 for each concentration). Photoacoustic monitoring of vessels on the mouse ear at 639 and 900 nm showed that at high concentrations the half-life of both nanoparticles in circulation was 15–20 min (Fig. 4a). At later times, rare flashes of photoacoustic signals appeared, preferentially from MNPs, which are likely associated with random fluctuation of nanoparticle number in the detected volume²⁰ and non-specific uptake of nanoparticles by blood cells (for example, blood-derived macrophages)^{29,30}. No photoacoustic signals were detected from either nanoparticle at a concentration of $\sim 1 \times 10^9$ nanoparticles ml⁻¹, suggesting that signals from unbound or non-specifically bound nanoparticles were below the background from blood. After intravenous injection of $\sim 1 \times 10^5$ cancer cells labelled with the nanoparticle cocktail *in vitro*, flashes of photoacoustic signals at both 639 and 900 nm with a dominant amplitude at 900 nm were observed immediately after injection (Fig. 4c) and then disappeared after 60–90 min (n = 3; green curve in Fig. 4b).

The occurrence of natural CTCs and their targeting *in vivo* were mimicked by intravenously injecting 1×10^5 unlabelled cancer cells in 50 ml PBS solution followed by injection of the GNT/MNP cocktail (n = 3). Flash photoacoustic signals at both 639 and 900 nm (similar to those in Fig. 4c) gradually increased within 8–10 min after injection of nanoparticles (red curve in Fig. 4b) to the approximate level of the photoacoustic signals observed from cells labelled *in vitro*, and then displayed a similar behaviour during clearance (red and green curves in Fig. 4b). Occasionally, we observed infrequent (a few per cent from the total number of signals) flashes of photoacoustic signals at 900 nm only (Fig. 4d) or at 639 nm only (Fig. 4e), which may be associated with targeting low numbers of CTCs that express one of the selected biomarkers. Blood produced weak background signals with consistent and comparable amplitudes at both wavelengths (Fig. 4f). We did not detect any photoacoustic signals with consistent amplitude above the background signal of the blood, indicating a negligible background from unbound nanoparticles. Attachment of a magnet immediately after injection of the nanoparticle cocktail revealed no signals over 3–5 min, but there was a gradual increase at an accelerated rate after 10 min at both wavelengths. These data suggest that we successfully targeted CTCs ~10 min after nanoparticle injection without any interference from unbound MNPs.

To detect CTCs originating from a primary tumour, 5×10^6 MDAMB-231 cells were inoculated subcutaneously into nude mice. At 2, 3 and 4 weeks of tumour development (Fig. 5a), a cocktail of the conjugated nanoparticles was injected intravenously into the circulation (n = 3 for each week). Two-colour photoacoustic detection of CTCs (Fig. 4c) at 20 min after injection (to allow clearance of most unbound nanoparticles, as shown in Fig. 4a) showed that the ratio of the numbers of CTCs in mouse ear to those in abdominal vessels (CTCs min⁻¹) increased from (0.9 ± 0.3)/(6 ± 2.1) at 2 weeks to (7.2 ± 0.3)/(26 ± 2.1) at 3 weeks and to (15.1 ± 2.7)/(47 ± 6.4) at 4 weeks. The numbers of CTCs (Fig. 5b) appear roughly correlated with the stage of the primary tumour progression shown in Fig. 5a and vessel sizes (Supplementary Fig. S3a,b). Attaching a magnet 20 min after injection of the nanoparticle cocktail into mice with a tumour at week 1 changed the character of the photoacoustic signal from infrequent flashes of signals to a continuous increase of photoacoustic signals (Fig. 5c). Similar patterns were observed after weeks 2 to 4. For example, the signal amplitude in the abdominal vessels of mice with a tumour at week 2 increased 88-fold within 1.5 h (Fig. 5d). Removal of the magnet led to release of the trapped CTCs bound to nanoparticles, yielding a

decrease in photoacoustic signal amplitudes. The partial decrease in amplitude is likely due to the remaining CTCs that are still adhered to the vessel wall.

Our findings suggest that duplex molecular targeting of CTCs with functionalized nanoparticles followed by their capture using dual magnetic–photoacoustic flow cytometry technology is feasible for the detection of CTCs in the bloodstream, *in vivo*, in real time. The application of two types of nanoparticles with strong magnetic (MNPs) and absorption (GNTs) properties allows us to combine effective magnetic enrichment of the low counts of CTCs in the bloodstream with highly sensitive and specific photoacoustic diagnosis of the accumulated cells, something that is difficult when using just one type of nanoparticle. MNPs and GNTs were used as mono-colour nanoparticles for duplex CTC targeting in this study, but the two absorption maxima of GNTs suggest their potential as two-colour nanoparticles²⁸.

A blood volume of ~5 l circulates through a human vein having a 2–3-mm diameter within 1 h (ref. 7). Hypothetically, patients may carry a magnet that is non-invasively attached to the skin above peripheral blood vessels for an hour or so, followed by rapid (minute-scale) photoacoustic detection of captured CTCs. Because CTCs can be concentrated from much larger volumes of blood *in vivo* when compared to the small blood samples taken for *in vitro* analysis (5–10 ml)^{2,3}, our *in vivo* approach has the potential to significantly improve sensitivity with a maximum of ~10³-fold gain (with an ultimate threshold of a few CTCs in whole blood) as determined by the ratio of the volume of blood *in vivo* to that *in vitro*. This makes it possible to use CTCs as markers for the early diagnosis of cancer. Once detected, the captured CTCs can be micro-surgically extracted for molecular and genetic tests or can be non-invasively ablated by laser^{6,22,28} in vessels or bypass to prevent metastasis.

In the future, the photoacoustic contrast of MNPs could be enhanced by developing gold–MNP complexes¹⁰, and magnetic properties could be introduced into GNTs by incorporating magnetic materials in them. Because targeting of CTCs in the circulation takes a matter of minutes due to the frequency of nanoparticle–cell collisions (see Supplementary Information), there is no requirement for nanoparticles that are able to circulate for long periods; on the contrary, fast clearance rates minimize background signals due to non-specific interaction of nanoparticles with normal cells and the potential toxic effects of nanoparticles on blood. This technology has the potential for clinical applications because MNPs, gold-based nanoparticles and photoacoustic technology have now been approved for pilot studies on humans^{28,31,32} (see Supplementary Information).

METHODS

Synthesis and bioconjugation of nanoparticles

MNPs from Ocean NanoTech, LLC, were bioconjugated as described previously^{26,27}, with some modifications (see Supplementary Information). GNTs as second photoacoustic molecular contrast agents were synthesized as described elsewhere with detailed reports on their physicochemical characteristics²⁸ (see Supplementary Information). GNTs with an average size of 12 × 98 nm² were selected for this study. The GNTs were conjugated with folic acid through electrostatic interactions. The resultant folate–GNT conjugate was washed three times in the presence of 1% polyethylene glycol (PEG). The GNTs were additionally conjugated with fluorescein (FITC) for verification by fluorescence imaging.

Cell labelling and characterization

Human breast cancer MDA-MB-231 cell line (ATCC) was cultured according to the standard procedure. Cells were incubated *in vitro* with serum free medium containing 20 pmol of bioconjugated MNPs at 37 °C for 2 h. The cells were washed with PBS three times.

The labelling efficiency was controlled by Prussian Blue staining, fluorescent imaging and magnetic resonance imaging (Fig. 2a,b; see Supplementary Information and Fig. S5), and by comparing photoacoustic signals from individual cells (total 300 cells for each experiment) at 639 and 900 nm. To verify labelling efficiency in the complex environment, mouse blood samples were spiked with MDA-MB-231 cells in various concentrations (10, 100 and 1,000 ml^{-1}) and treated with 1 and 0.1 nM unconjugated or conjugated GNTs and MNPs, alone or in a cocktail (20:80), at 37 °C for 0.1, 0.5, 4 and 12 h under static and flow conditions.

Experimental animals

Nude mice nu/nu (weighing 20–25 g) purchased from Harlan Sprague–Dawley were used in accordance with protocols approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Experiments were performed on thin (~250 mm) mouse ear with well-distinguished blood microvessels (Supplementary Fig. S3a) at a depth of 50–100 mm and with a diameter of 50–100 mm with blood velocities of 3–7 mm s^{-1} , and on blood vessels with a diameter of 200–300 mm in the abdominal area of the mouse (Supplementary Fig. S3b) at a depth of 0.3–0.5 mm and flow velocity of 1–3 cm s^{-1} . After standard anaesthesia (ketamine/xylazine, 50/10 mg kg^{-1}), each animal was placed on the customized heated microscope stage. Breast tumours were created in the back flank area of mice by inoculating with 5×10^6 MDA-MB-231 cells in 50 ml of PBS.

Photoacoustic instrumentation with magnetic module

The integrated setup was built as described previously^{6,21,22} on the technical platform of an Olympus BX51 microscope (Olympus America) and a tunable optical parametric oscillator (OPO, Lotis) with a pulsewidth of 8 ns, a repetition rate of 10–50 Hz, a wavelength in the range 420–2,300 nm and a fluence range of 1–10,000 mJ cm^{-2} (Supplementary Fig. S1). In addition, a diode laser 905-FD1S3J08S (Frankfurt Laser Company) with driver (IL30C, Power Technology) was used with a wavelength of 905 nm, pulse width of 15 ns and a pulse repetition rate of 10 kHz. Delivery of laser radiation to blood vessels was performed either with a microscope schematic (Fig. 1a; Supplementary Figs S1–S3) or using a 330-mm fibre with focusing tip (Fig. 1a; Supplementary Fig. S3). Laser-induced photoacoustic waves were detected using an ultrasound transducers (model 6528101, 3.5 MHz, 4.5 mm in diameter; Imasonic), amplified (amplifier 5660B, 2 MHz, gain 60 dB; Panametrics), recorded with a Tektronix TDS 3032B oscilloscope and a Boxcar (Stanford Research Systems), and analysed with standard and customized software. The setup was equipped with a high-speed (200 MHz) analog-to-digital converter board (National Instruments Corp., PCI-5152, 12-bit card, 128 MB of memory), specialized software (LabVIEW; National Instruments) and a Dell Precision 690 workstation with a quadcore processor, 4 GB of RAM and Windows Vista 64 bit operating system. The transducer was gently attached to skin or samples or slides, and warmed water or conventional ultrasound gel was topically applied for better acoustic matching between the transducer and the samples. In two-colour modes, in addition to an OPO pulse with selected wavelength of 900 nm, a second co-linear probe pulse was used from a Raman shifter with a wavelength of 639 nm, pulse duration of 12 ns and a 10-ms delay to the pump pulse. The permanent magnetic field was provided by a cylindrical neodymium–iron–boron (NdFeB) magnet with nickel–copper–nickel coating, 3.2 mm in diameter and 9.5 mm long, and with a surface field strength of 0.39 T (MAGCRAFT). In selected experiments the magnet was used with a custom-made 0.7-mm hole through which to deliver laser radiation by means of a fibre (Fig. 1a; Supplementary Fig. S3e). The distances between the magnet and examined vessels ranged from 50 to 100 mm (mouse ear) or 0.3 to 0.5 mm (abdominal area).

Statistical methods

Results were expressed as means plus/minus the standard error of at least three independent experiments ($P, 0.05$). Statistica 5.11 software (StatSoft) and MATLAB 7.0.1 (MathWorks) were used for the statistical calculations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported in part by National Institute of Health grant numbers R01EB000873, R01CA131164, R01EB009230, R21EB0005123 and R21CA139373 (V.P.Z.), National Cancer Institute grant number CA133722 (L.Y.), National Science Foundation grant numbers DBI-0852737 (V.P.Z.) and CMMI-0709121 (J.-W.K.) and the Arkansas Biosciences Institute (J.-W.K. and V.P.Z.). The authors would like to thank Y.A. Wang of Ocean Nanotech, LLC, for providing MNPs, H.-M. Moon for his assistance with GNT synthesis, J. Daniels for cell culturing, Z. Cao for bioconjugation of MNPs and cell labelling, H.K. Sajja for production of human amino-terminal fragments of urokinase plasminogen activator, H. Mao for magnetic resonance imaging and S. Fergusson for his assistance with laser measurements.

References

1. Christofori G. New signals from the invasive front. *Nature*. 2006; 441:444–450. [PubMed: 16724056]
2. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nature Rev Cancer*. 2008; 8:329–340. [PubMed: 18404148]
3. Nagrath S, et al. Isolation of rare circulating tumor cells in cancer patients by microchip technology. *Nature*. 2007; 450:1235–1239. [PubMed: 18097410]
4. Riethdorf S, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clin Cancer Res*. 2007; 13:920–928. [PubMed: 17289886]
5. Georgakoudi I, et al. In vivo flow cytometry: a new method for enumerating circulating cancer cells. *Cancer Res*. 2004; 64:5044–5047. [PubMed: 15289300]
6. Zharov VP, Galanzha EI, Shashkov EV, Khlebtsov N, Tuchin V. In vivo photoacoustic flow cytometry for monitoring circulating single cancer cells and contrast agents. *Opt Lett*. 2006; 31:3623–3625. [PubMed: 17130924]
7. He W, Wang H, Hartmann LC, Cheng JX, Low PS. In vivo quantitation of rare circulating tumor cells by multiphoton intravital flow cytometry. *Proc Natl Acad Sci USA*. 2007; 104:11760–11765. [PubMed: 17601776]
8. Heyn C, et al. In vivo magnetic resonance imaging of single cells in mouse brain with optical validation. *Magn Reson Med*. 2006; 55:23–29. [PubMed: 16342157]
9. Medarova Z, Pham W, Kim Y, Dai G, Moore A. In vivo imaging of tumor response to therapy using a dual-modality imaging strategy. *Int J Cancer*. 2006; 118:2796–2802. [PubMed: 16385568]
10. Larson TA, Bankson J, Aaron J, Sokolov K. Hybrid plasmonic magnetic nanoparticles as molecular specific agents for MRI/optical imaging and photothermal therapy of cancer cells. *Nanotechnology*. 2007; 18:325101.
11. Kim DH, Kim KN, Kim KM, Lee YK. Targeting to carcinoma cells with chitosan-and starch-coated magnetic nanoparticles for magnetic hyperthermia. *J Biomed Mater Res A*. 2009; 88:1–11. [PubMed: 18257079]
12. McCarthy JR, Kelly KA, Sun EY, Weissleder R. Targeted delivery of multifunctional magnetic nanoparticles. *Nanomedicine*. 2007; 2:153–167. [PubMed: 17716118]
13. Muthana M, et al. A novel magnetic approach to enhance the efficacy of cell based gene therapies. *Gene Ther*. 2008; 15:902–910. [PubMed: 18418420]
14. Polyak B, et al. High field gradient targeting of magnetic nanoparticle-loaded endothelial cells to the surfaces of steel stents. *Proc Natl Acad Sci USA*. 2008; 105:698–703. [PubMed: 18182491]

15. Xia N, et al. Combined microfluidic–micromagnetic separation of living cells in continuous flow. *Biomed Microdev.* 2006; 8:299–308.
16. Benez A, Geiselhart A, Handgretinger R, Schiebel U, Fierlbeck G. Detection of circulating melanoma cells by immunomagnetic cell sorting. *J Clin Lab Anal.* 1999; 13:229–233. [PubMed: 10494132]
17. Pamme N. Magnetism and microfluidics. *Lab Chip.* 2006; 6:24–38. [PubMed: 16372066]
18. Zhang HF, Maslov K, Stoica G, Wang LV. Functional photoacoustic microscopy for high-resolution and noninvasive in vivo imaging. *Nature Biotechnol.* 2006; 24:848–851. [PubMed: 16823374]
19. Zerda A, et al. Carbon nanotubes as photoacoustic molecular imaging agents in living mice. *Nature Nanotech.* 2008; 3:557–562.
20. Zharov VP, et al. Photoacoustic flow cytometry: principle and application for real-time detection of circulating single nanoparticles, pathogens and contrast dyes in vivo. *J Biomed Opt.* 2007; 12:051503. [PubMed: 17994867]
21. Galanzha EI, Shashkov EV, Tuchin VV, Zharov VP. In vivo multispectral, multiparameter photoacoustic lymph flow cytometry with natural cell focusing, label-free detection and multicolor nanoparticle probes. *Cytometry.* 2008; 73:884–894. [PubMed: 18677768]
22. Galanzha EI, Shashkov EV, Spring PM, Suen JY, Zharov VP. In vivo, noninvasive label-free detection and eradication of circulating metastatic melanoma cells using two-color photoacoustic flow cytometry with a diode laser. *Cancer Res.* 2009; 69:7926–7934. [PubMed: 19826056]
23. Kämmerer U, Thanner F, Kapp M, Dietl J, Sütterlin M. Expression of tumor markers on breast and ovarian cancer cell lines. *Anticancer Res.* 2003; 23:1051–1055. [PubMed: 12820346]
24. Stoeva SI, Lee JS, Smith JE, Rosen ST, Mirkin CA. Multiplexed detection of protein cancer markers with biobarcode nanoparticle probes. *J Am Chem Soc.* 2006; 128:8378–8379. [PubMed: 16802785]
25. Li PC, et al. Photoacoustic imaging of multiple targets using gold nanorods. *IEEE Trans Ultrason Ferroelectr Freq Control.* 2007; 54:1642–1647. [PubMed: 17703668]
26. Yang L, et al. Development of receptor targeted magnetic iron oxide nanoparticles for efficient drug delivery and tumor imaging. *Special Issue on Cancer Nanotechnology. J Biomed Nanotechnol.* 2008; 4:439–449.
27. Yang L, et al. Receptor-targeted nanoparticles for in vivo imaging of breast cancer. *Clin Cancer Res.* 2009; 15:4722–4732. [PubMed: 19584158]
28. Kim JW, Galanzha EI, Shashkov EV, Moon HM, Zharov VP. Golden carbon nanotubes as multimodal photoacoustic and photothermal high contrast molecular agents. *Nature Nanotech.* 2009; 4:688–694.
29. Bulte JW, Kraitchman M. Iron oxide MR contrast agents for molecular and cellular imaging. *NMR Biomed.* 2004; 17:484–499. [PubMed: 15526347]
30. Clement JH, et al. Different interaction of magnetic nanoparticles with tumor cells and peripheral blood cells. *J Cancer Res Clin Oncol.* 2006; 132:287–292. [PubMed: 16432758]
31. Petrov YY, Petrova IY, Patrikeev IA, Esenaliev RO, Prough DS. Multiwavelength optoacoustic system for noninvasive monitoring of cerebral venous oxygenation: a pilot clinical test in the internal jugular vein. *Opt Lett.* 2006; 31:1827–1829. [PubMed: 16729084]
32. Ermilov SA, et al. Laser optoacoustic imaging system for detection of breast cancer. *J Biomed Opt.* 2009; 14:024007. [PubMed: 19405737]

Figure 1. In vivo magnetic enrichment using two-colour photoacoustic detection of CTCs

a, Schematic showing the detection setup. The laser beam is delivered either close to the external magnet or through a fibre-based delivery system. **b**, Schematic (left) and transmission electron microscopy image (right) of MNPs, each with a 10-nm core, a thin (~2 nm) layer of amphiphilic triblock copolymers modified with short polyethylene glycol (PEG) chains and the amino-terminal fragment (ATF) of the urokinase plasminogen activator. Scale bar, 10 nm. **c**, Schematic (left) and topographic atomic force microscopy image (right) of a GNT (12 nm × 98 nm) coated with PEG and folic acid. **d**, Photoacoustic spectra of ~70-mm veins in mouse ear (open circles). The average standard deviation for each wavelength is 18%. Absorption spectra of MNPs and GNTs (dashed red and green curves) are normalized to photoacoustic signals from CTCs labelled with MNPs (filled red circle) and GNTs (filled green circle).

Figure 2. In vitro measurement under stationary conditions

a, Prussian blue staining of MDA-MB-231 cells incubated with non-conjugated MNPs (left) and amino-terminal fragment MNPs (right) for 2 h at 37°C. **b**, Fluorescence images of a single cell incubated with FITC-GNTs (left) and folate-FITC-GNTs (right) for 30 min at 37°C. **c**, Photoacoustic signals from 10-nm MNPs ($\sim 1 \times 10^{11}$ nanoparticles mL^{-1}) without a magnet (red curve with filled squares) and a single MNP-labelled cell in a suspension placed on a 120- μm -thick microscope slide with (blue curve with filled circles) and without (green curve with open circles) a magnet. The average standard deviation for each laser fluence is 32%. **d**, Photoacoustic signals from nanoparticles at different concentrations. The background level is from mouse blood. The error bars represent standard error.

Figure 3. In vitro measurement under flow conditions

a, Schematic showing the in vitro testing tube. **b–d**, Magnetic capturing of MNP-labelled cancer cells in PBS at a flow velocity of 5 mm s⁻¹ (**b**) and of labelled cancer cells in the presence of free MNPs at 0.1 cm s⁻¹ (**c**) and 5 cm s⁻¹ (**d**). **e,f**, Photoacoustic signals from captured cells (**e**) and surrounding medium (**f**) in **b**. Amplitude/time scales: 500 mV/div and 4 ms/div (**e**) and 50mV/div and 4 ms/div (**f**). **g**, Capturing efficiency of MNP-labelled cells and free MNPs calculated as the relative amount of captured cells and MNPs using the optical images in Fig. 3b–d and Supplementary Fig. S6–b d at different flow velocities normalized to those at a velocity of 0.1 cm s⁻¹. The average standard deviation for each velocity is 26%. The error bars represent the standard error.

Figure 4. In vivo measurements of nanoparticles and cells mimicking CTCs

a, Kinetics of clearance of 30-nm MNPs (red curve with filled circles) and GNTs (blue curve with open circles) at a concentration of 1×10^{11} nanoparticles ml^{-1} in a ~70-mm mouse ear vein. **b**, Photoacoustic monitoring of CTCs in the abdominal vessel using fibre-based photoacoustic flow cytometry. The graph shows the clearance for cells that were labelled with the nanoparticle cocktail in vitro before injection (green curve with filled circles) and those labelled with the nanoparticle cocktail in vivo after sequential injections of unlabelled cells alone and then nanoparticles alone (red curve with open circles). GNT/MNP cocktails had a GNT/MNP ratio of 20:80 in 10ml PBS ($n=3$). The average standard deviation for each wavelength is 28%. **c–e**, Typical photoacoustic signals at different wavelengths from CTCs labelled with MNPs and GNTs (**c**), GNTs only (**d**) and MNPs only (**e**). **f**, Photoacoustic signals from blood vessels only. The error bars in **a** and **b** represent standard error.

Figure 5. Photoacoustic detection and magnetic enrichment of CTCs in tumour-bearing mice
a, The size of the primary breast cancer xenografts at different stages of tumour development. **b**, The average rate of CTCs in mouse ear vein over a period of several weeks. **c**, Photoacoustic signals from CTCs in abdominal skin vessels obtained with fibre schematics at week 1 of tumour development before and after magnet action. The average standard deviation for each wavelength is 24%. **d**, Photoacoustic signals from CTCs in abdominal skin vessels before, during (3 min) and after magnetic action at week 2 of tumour development. The error bars in **b** and **d** represent standard error.

Table 1

Labelling efficiency of breast cancer cells (MDA-MB-231) (1 h, 37 °C) in static condition *in vitro*, obtained with a photoacoustic cytometer.

Samples	Nanoparticles (%)					
	MNPs	GNTs	MNPs+GNTs	ATF-MNPs	Folate-GNTs	ATF-MNPs + Folate-GNTs
Cells in PBS	5	15	18	85	89	98
Cells in mouse blood	3	8	11	71	76	96
Normal mouse blood	5	4	8	6	5	9

MNP, magnetic nanoparticle; GNT, golden carbon nanotube; ATF, amino-terminal fragment; PBS, phosphate buffer saline.