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HER-2/neu Targeted Delivery of a Nanoprobe Enables Dual Photoacoustic and Fluorescence Tomography of Ovarian Cancer

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Abstract

Development of sensitive and specific imaging approaches for the detection of ovarian cancer holds great promise for improving survival of ovarian cancer patients. Here we describe a dual-modality photoacoustic and fluorescence molecular tomography (PAT/FMT) approach in combination with a targeted imaging probe for three-dimensional imaging of ovarian tumors in mice. We found that the selective accumulation of the HER-2/neu targeted magnetic iron oxide nanoparticles (IONPs) led to about 5-fold contrast enhancements in the tumor for PAT, while near-infrared (NIR) dye labeled nanoparticles emitted strong optical signals for FMT. Both PAT and FMT were demonstrated to be able to detect ovarian tumors located deep in the peritoneal cavity in mice. The targeted nanoprobe allowed mapping tumors in high resolution via PAT, and with high sensitivity and specificity via FMT. This study demonstrated the potential of the application of HER-2/neu-targeted PAT/FMT approach for non-invasive or intraoperative imaging of ovarian cancer.

Keywords

Photoacoustic tomography; Fluorescence molecular tomography; HER-2/neu targeted nanoparticles; Iron oxide nanoparticle

Background

Ovarian cancer is considered as a “silent killer” due to lack of effective approaches for early detection and therapy. Most ovarian cancer patients are diagnosed at the advanced stage and don’t respond well to conventional therapeutics. The mortality rate of the ovarian cancer remains one of the worst among all cancer types.^{1, 2} Ultrasound (US), magnetic resonance imaging (MRI) and computed tomography (CT) are the most widely used clinical imaging systems for the detection of ovarian cancer. However, these traditional imaging modalities depend on morphological characteristics and have low specificity and sensitivity in detection small tumor lesions.^{3–5} Over the past several years, there has been a noteworthy interest in

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the use of imaging probes that specifically target ovarian cancer cells for enhancing sensitivity and specificity in tumor detection. Positron emission tomography (PET) and single photon emission tomography (SPECT) in combination with targeted image probes have been used clinically to detect various cancers with high specificity and sensitivity. However, those imaging approaches have a low resolution to precisely locate tumor lesions in the peritoneal cavity.^{6–8} Therefore, there is an urgent need to develop noninvasive approaches with high resolution, sensitivity, and specificity for the detection of ovarian cancer.

Photoacoustic tomography (PAT) has characteristics that are well-suited for the development of a new tumor imaging system with a good resolution and imaging depth. It is a hybrid imaging method that combines optical contrast with the high resolution of ultrasound imaging that makes it suitable for various applications in ophthalmology, dermatology, gastroenterology, and breast imaging.^{9–14} Recently, PAT contrast agents have been investigated to enhance the contrast for imaging brain tumors,¹⁵ angiogenesis,¹⁶ melanomas,¹⁷ lymph nodes,^{18, 19} and the cerebral cortex.²⁰ Of all the available contrast agents, iron oxide nanoparticle (IONP) is considered as a potential PAT agent because of the following properties: a) a modest light absorption in near-infrared wavelengths;²¹ b) biodegradable and biocompatible with multifunctional characters; and c) ability of carrying therapeutic agents for the production of theranostic nanoparticles for PAT monitoring drug delivery and tumor responses to therapies. IONP-mediated photoacoustic effect was used for the detection of circulating tumor cells in the blood of tumor-bearing mice.²² Results of our study also showed the contrast enhancement in breast cancer lesions for PAT imaging after systemic delivery of a receptor targeted IONPs.²¹ Therefore, IONPs have potential for the development of PAT contrast agents. However, PAT has relatively low sensitivity compared to pure optical imaging due to the limitations of acoustic transducers.²³ Additionally, the presence of endogenous photoacoustic signals in some normal organs, such as the kidney, liver, spleen and heart, may interfere with specificity of PAT imaging.

Fluorescence molecular tomography (FMT) is an inexpensive and fast three-dimensional (3D) optical imaging modality that has been used for molecular imaging by various groups.²⁴ In contrast to conventional two-dimensional (2D) fluorescence molecular imaging, FMT provides improved localization and quantification in deep tissues.^{23–25} Previously, we reported that FMT was more sensitive and specific for the detection of low concentration contrast agents in tissues compared with PAT.²³ However, FMT has limited spatial resolution. Hence the combination of these two modalities with targeted nanoprobe should allow noninvasive imaging of ovarian tumors with high resolution, specificity and sensitivity.

Results of our recent study have shown that near-infrared dye labeled Z_{HER2:342} conjugated IONPs (NIR-830-Z_{HER2:342}-IONP) specifically targeted to primary and metastatic tumors in an orthotopic human ovarian cancer xenograft model and produced strong imaging signals for 2D-optical and magnetic resonance imaging.²⁶ In this study, we wanted to determine the feasibility of application of this HER-2 targeted IONP as a multimodality imaging probe for the detection of ovarian cancer using FMT and PAT imaging device developed by our group.

Methods

Cell lines

The human ovarian cancer cell line SKOV3, stably expressing a firefly luciferase gene (SKOV3-luc) was provided by Dr. D. Matei at Indiana University Purdue University at Indianapolis (IUPUI). SKOV3-luc cells were cultured in McCoy's 5A (Cellgro, Mediatech

Inc. VA, USA) supplemented with 10% fetal bovine serum (Hyclone from Thermo scientific) and 1% penicillin streptomycin (Hyclone, Logan, Utah). Cultured cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

HER-2/neu specific affibody conjugation to IONP

A histidine-tagged HER-2-specific affibody (His6-Z_{HER2:342}-Cys), was provided by Dr. J. Capala at the National Institute of Biomedical Imaging and Bioengineering (NIBIB), Bethesda, Maryland.²⁷ Z_{HER2:342} is a 58 amino acid scaffold protein with a molecular weight of ~ 6.5 kDa. The NIR dye, NIR-830 maleimide, which was developed by our group with excitation and emission spectrum of 797/815 nm (Figure 1, A) was used for site specific labeling of the affibody. Iron oxide nanoparticles with a core size of 10 nm were obtained from Ocean Nano Tech, LLC (Springdale, AR) and coated with amphiphilic polymers by using established protocols.²⁸ The amphiphilic polymers have carboxyl groups for bio-conjugation with amine groups of Z_{HER2:342}-NIR-830 (Figure 1, B). Briefly, the Z_{HER2:342} which contains a unique C-terminal cysteine residue for thiol reactive maleimide dye labeling was subjected to reduction with 5mM (mmol/L) TCEP for 15 minutes at room temperature. The maleimide form of NIR-830 dye was then added to Z_{HER2:342}-TCEP mixture (Z_{HER2:342}: dye molar ratio 1:4) for overnight at 4° C. Finally, the NIR dye-affibody conjugated to the IONPs using ethyl-3-dimethyl amino propyl carbodiimide (EDAC, Sigma-Aldrich) and N-hydroxy-sulfo-succinimide (sulfo-NHS, Sigma-Aldrich) according to the carbodiimide method for overnight at 4°C (IONP: Z_{HER2:342} molar ratio 1:10). Briefly, 100 nmol of EDAC and 200 nmol of sulfo-NHS were added to the aqueous solution of 1 mg of IONPs, allowing the activation of IONPs for 15 minutes in Borate buffer at pH 5.5. The reaction was terminated by removing the buffer through a brief centrifugation using 100 K Nanosep column (Pall Corporation, Port Washington, NY), and then adding 10 mM Borate buffer, pH 8.5. After a brief wash with Borate buffer, pH 8.5, the reaction of IONPs with Z_{HER2:342}-NIR-830 dye was carried out for 4–6 hours at room temperature. The final product NIR-830-Z_{HER2:342}-IONP was made after washing the mixture through Nanosep 100 K column for 3 times and conjugate was stored in 10 mM Borate buffer at pH 8.5.

Preparation of NIR-bovine serum albumin -IONP (NIR -830-BSA-IONPs)

Bovine serum albumin (BSA) was used to produce control non-targeted IONPs. Briefly, 1mg of BSA (Sigma), at the molar ratio of 1:4, was added to 60 nmol of NIR-830-maleimide dye and allowed for conjugation for 4 hours at room temperature or overnight at 4° C. After washing the BSA-dye complex with a 3K spin column, the complex was allowed for conjugation with activated IONPs at the molar ratio of 1:10 (IONP: BSA). The final NIR-830-BSA-IONPs conjugate were purified using Nanosep 100K column filtration. The hydrodynamic sizes of IONP, NIR-830-Z_{HER2:342}-IONP and NIR-830-BSA-IONP used for the experiments were measured by dynamic light scattering (DLS) instrument (Zeta-sizer Nano: ZS90, Malvern Instruments Ltd.).

Evaluation of labeling efficiency by Prussian Blue Staining

Approximately 10⁵ of SKOV3 cells were seeded in each cell culture chamber for overnight. 100 nM of NIR-830-Z_{HER2:342}-IONP, NIR-830-BSA-IONP or IONPs were added into the RPMI1640 containing 2% FBS culture media and then incubated at 37°C for 2–3 hours. The medium containing particles were then removed and cells were washed with PBS for 3 times followed by fixation with 4% paraformaldehyde for 20 minutes. After a brief wash with PBS, Prussian blue staining solution prepared by 1:1 mixture of 5% potassium ferrocyanide and 5% HCl acid was added for 30 minutes to 2 hours at 37°C to confirm the presence of intracellular IONPs under optical microscope (400X magnification). Finally the percentage

of iron positive cells was obtained by counting ~ 200 number of cells. Every time when the fresh conjugates were prepared, specificity of the conjugates was tested in different passages of the SKOV3-luc cells.

Orthotopic human ovarian cancer xenograft model

The ovarian tumor model was established by injecting 5×10^4 of SKOV3 luciferase gene positive human ovarian cancer cells into the ovaries of 6–8 week old female athymic nude mice (Harlan). All surgical and imaging procedures were approved by Emory University Institutional Animal Care and Use Committee (IACUC). Mice were imaged when orthotopically xenografted tumors reached around 5mm in size, usually 4–6 weeks after the tumor cell injection.

Fluorescence molecular tomography imaging system

A handheld optical fiber array based FMT system is shown in Figure S1, A. In this system, a continuous-wave (CW) 785nm laser was mounted in a 2D linear stage. A convex lens was used to couple the laser beam into each source fiber in the array. For each source illumination, a 1024×1024 pixels CCD camera (Princeton Instruments, Trenton NJ) equipped with a band-pass filter was used to collect the emitted light from the detection interface. Optimal exposure time was selected for each experiment and binning of 4×4 pixels was used to improve the signal-to-noise ratio (SNR). A photograph of the imaging detector consisting of 10 sources and 15 detectors is shown in Figure S1, A. Fluorescence images were reconstructed using an iterative finite element-based algorithm, described by Zhao et al.²⁹

Photoacoustic imaging system

A schematic of the photoacoustic imaging system is shown in FigureS1, B. A tunable Ti:Sapphire laser (LT-2211A, LOTIS TII) with 8–30 ns pulse duration and 10 Hz repetition rate was used. The laser beam was expanded, split and coupled into two optical fiber bundles adjusted to allow optimal illumination within the imaging area (20×20 mm²). The measured photon energy density on the tissue surface was 8 mJ/cm². A 3.5 MHz focused ultrasound transducer (V383, Olympus) with 15 mm aperture and 35 mm focal length was used to receive laser-induced photoacoustic waves, yielding axial and lateral resolutions of 400 μm and 820 μm, respectively. The imaging probe, mounting transducer and optical fiber bundles, was fixed onto a 2D moving stage. One-dimensional (1D) depth-resolved images (A-lines) at each transducer location were acquired through raster scanning along one transverse direction, and formed 2D images (B-scans). Further raster scanning along the other transverse direction enabled the formation of 3D images. The total raster scanning points was 100 A-lines along each direction with an interval of 200 μm over a distance of 20 mm. It took 20 minutes for one scan and most 3D PAT images are shown in maximum amplitude projection (MAP) form.

In vivo planar fluorescence imaging

Planar fluorescence imaging was performed 24 and 48 hours after systemic delivery of NIR-830-Z_{HER2:342}-IONP using a Kodak *in vivo* imaging system FX (Carestream Health Inc., New Haven, CT) to demonstrate specific accumulation of the targeted IONPs in the ovarian tumors, and anatomically localize the tumors.²⁶ Briefly, an 800 nm excitation filter and an 850 nm emission filter were used. The imaging system also includes F-stop 2.5 and 100 mm field of view (FOV). The *in vivo* images were captured by an excitation time of 3 minutes with a gamma value of 0.2. By using the built-in Kodak software, the mean fluorescence intensity (MFI) was measured over a region of interest (ROI) in the tumor area

and the surrounding skin area, and the corresponding ratio was calculated as the signal to body background ratio (SBR).

In vivo imaging in animal tumor models

Bioluminescence images were carried out weekly after the injection of 30 mg/kg luciferin substrate in the anesthetized mice to track the SKOV3-luc orthotopic tumor growth using the IVIS Imaging System (Xenogen) (see Figure S 2). Acquisition times started with 1 minute and were reduced later onto avoid signal saturation. Signal strength was quantified with LIVINGIMAGE software (Xenogen) by measuring photon flux over a ROI.

Three groups of mice were examined using both FMT and PAT. The first group (n=4) received an injection of 400 picomole of NIR-830-Z_{HER2:342}-IONP. The second group (n=3) received an injection of 400 picomole of NIR-830-BSA-IONP. The third group (n=3) without injection was used as a control.

Histology analysis

Tumors along with normal organs were collected after sacrificing the mice. The tissues were fixed with 10% buffered formalin. Paraffin tissue sections were stained with Prussian blue or hematoxylin and eosin (H&E). Images were acquired at 200X magnification using a Zeiss Axioplan 2 upright microscope.

Statistical analysis

All data used for statistical analysis were summarized using means $\pm$ standard error of the mean (SEM).

Results

Taking advantage of smaller size and highly affinity nature of the affibody, we have used a relatively new class of affinity molecule, HER-2 affibody (Z_{HER2:342}), for conjugating to IONPs. Prior to the conjugation, Z_{HER2:342} was pre-labeled with a near infrared dye (NIR-830 dye) to the cysteine residue of the affibody molecule. The bi-conjugate NIR-830-Z_{HER2:342} was finally coupled to the carboxyl group of the amphiphilic polymer coating of IONP. Figure 1, B depicts the schematic representation of the production of the imaging probe, NIR-830-Z_{HER2:342}-IONP. By using Zeta-sizer Nano, the hydrodynamic size of the IONP was 14.7 ± 3.58 nm and increased upon conjugating with Z_{HER2:342} (NIR-830-Z_{HER2:342}-IONP: 21.6 ± 5.61 nm) and BSA (NIR-830-BSA-IONP: 26.12 ± 2.22 nm). Specific binding and uptake efficiency of the targeted nanoparticles (NIR-830-Z_{HER2:342}-IONP) were examined in the HER-2 over expressing SKOV3 cells as shown in Figure 1, C. In general, when 100 nM of Fe equivalent concentration of targeted IONPs were incubated with SKOV3 cells for 5 hours followed by 1 hour Prussian blue stain, about 60% cells were blue stained iron positive cells. The iron stained cells increased with higher concentrations of targeted nanoparticles. However cellular uptake by non-targeted IONPs was significantly lower, when SKOV3 cells were incubated with non-targeted IONPs under similar conditions (Figure 1, C).

The absorbance spectrum of IONPs and NIR-830-Z_{HER2:342}-IONP used for photoacoustic imaging was evaluated (see Part I in supplementary materials) within NIR range and shown in Figure 1, D, where absorption of IONPs decreased as the wavelength increased. From the spectrum, we noted that even the absorption of NIR-830-Z_{HER2:342}-IONP had a peak value at 820 nm contributed by the NIR dye, its absorption at 730nm was still higher than that at 820nm since IONPs were likely to be the major contributor to the photoacoustic enhancement due to a low concentration of NIR-830 dye molecules on each IONP (10 dye

molecules per IONP). To verify the assumption, we compared the contribution to the photoacoustic effect by pure NIR-830 dye and NIR-830-Z_{HER2:342}-IONP (see Part I in supplementary materials). From the results shown in Figure 1, E, we noticed that when the concentration of NIR-830 dye was 40 times higher than the concentration of IONPs in NIR-830-Z_{HER2:342}-IONP, they contributed equally to the photoacoustic effects. That means one IONP equals to 30 dye molecules in the generation of photoacoustic signals with consideration of 10 dye molecules per IONP in NIR-830-Z_{HER2:342}-IONP.

To obtain the equivalent concentration of IONPs in NIR-830-Z_{HER2:342}-IONP to normal ovarian tumors in photoacoustic imaging, we compared the photoacoustic signals of different concentrations of IONPs in NIR-830-Z_{HER2:342}-IONP with that of the tumors without any contrast agents (see Part I in supplementary materials). Photoacoustic signals decreased as expected when the concentration of IONPs in NIR-830-Z_{HER2:342}-IONP decreased. When the concentration of IONPs reduced to 0.1 picomole/ μ L, respectively, the amplitude of photoacoustic signals becomes almost the same as that from the tumor (Figure 1, E). The amplitude change of photoacoustic signals relevant to the concentrations of IONPs in NIR-830-Z_{HER2:342}-IONP was quantitatively plotted in Figure 1, F. A linear regression fit of the data yielded an R^2 equaling to 0.99, which was expected given the amplitude of the photoacoustic signal was proportional to the concentration of IONPs in NIR-830-Z_{HER2:342}-IONP.

To compare the sensitivity of FMT and PAT in imaging when NIR-830-Z_{HER2:342}-IONP produced contrasts, we performed planar fluorescence and FMT phantom experiments using different concentrations of IONPs in NIR-830-Z_{HER2:342}-IONP (see Part II in supplementary materials). We found that when the concentration reduced to 0.04 picomole/ μ L, they were still detectable for both planar fluorescence imaging and FMT (Figure S3). In the case of photoacoustic imaging, there was no enhancement when the concentration became as low as 0.1 picomole/ μ L. Results of our study suggested that our handheld FMT has higher sensitivity compared to PAT for imaging NIR-830-Z_{HER2:342}-IONP.

From *in vivo* planar fluorescence imaging, it showed that the fluorescence intensity reached to the strongest level in the ovarian cancer at 48 hours following systemic delivery (Figure S4) when the tumor size was < 5mm.²⁶ However, in tumor-bearing mice without receiving injection of the imaging probes or injected with NIR-830-BSA-IONP, no significant imaging signals were detected in the tumor region. The mean fluorescence intensity (MFI) of the tumor for the animal groups that received injection of NIR-830-Z_{HER2:342}-IONP or NIR-830-BSA-IONP 24 and 48 hours post injection are plotted in Figure 2, A. The high level of the MFI found in the tumors of the mice that received NIR-830-Z_{HER2:342}-IONP suggested effective accumulation of the nanoprobes in the tumor at both time points.

Four time points were selected to perform photoacoustic imaging and quantitative analysis was shown in Figure 2, B. Photoacoustic contrasts in tumors of mice before injection of imaging probes were comparable. 24 hours post-injection, the contrast of tumors of mice that received NIR-830-Z_{HER2:342}-IONP increased significantly and reached the peak value 48 hours post injection, which is consistent with the planar fluorescence imaging. However, no significant contrast enhancement appeared in non-targeted and non-injection groups.

All three groups of mice were imaged by both PAT and FMT. The imaging area was guided by both bioluminescence imaging (BLI) (Figure 2, C) and planar fluorescence imaging (Figure 2, D). As expected, tumors of the mice that received injection of NIR-830-Z_{HER2:342}-IONP had the strongest photoacoustic signal (left image in Figure 2, E), compared with the tumors of the mice that received injection of NIR-830-BSA-IONP (middle image in Figure 2, E) or mice in the no injection control group (right image in

Figure 2, *E*). The corresponding FMT results were shown in Figure 2, *F*, which was consistent with the results of planar fluorescence imaging and PAT. Data from multi-spectral plots shown in Figure 2, *G* revealed that the strongest photoacoustic signal in the tumors of mice that received injection of NIR-830-Z_{HER2:342}-IONP was at 730 nm, which was 500% higher than that of non-injection controls, and 300% greater than that of the mice injected with NIR-830-BSA-IONP.

After the mice were sacrificed, tumors as well as normal organs such as the liver, kidney, spleen and heart in targeted and non-targeted groups were resected and evaluated by planar fluorescence imaging, PAT and FMT. In fluorescence imaging, we found strong optical signals in the kidney and liver but not in other normal organs (Figure 3, *A* and *B*). However, in PAT images, the tumor of targeted group and hearts emitted strongest photoacoustic signals and the photoacoustic signals of the spleen were stronger than that of the liver and kidney. From histological sections and quantitative analysis in Figure 4, systemic accumulation of IONPs in the tumor led to strongest photoacoustic emission, non-specific uptake of the IONP by the reticuloendothelial system (RES) in the liver and spleen resulted in a higher photoacoustic emission compared with that of the kidney. A large volume of blood in the heart generated stronger photoacoustic signals than other normal organs since the light absorption of blood at 730 nm is stronger than that of IONPs.²¹ Additionally, we did see strong optical signals of the kidney, but didn't detect blue iron stained cells in the tissue section of the kidney. This may be caused by the renal clearance of free dye molecules or breakdown products of targeting ligands in the kidney to retain the nanoparticles in the tissue section.²⁶

To evaluate the clinical potential of PAT/FMT technique for intraoperative imaging of ovarian tumors in humans, we examined the ability of imaging of tumors located in deep tissues by adding different thicknesses of chicken breast tissues on the top of mouse back. The top row in Figure 5, *A* showed the B-scan PAT images with the different thicknesses of the chicken breast. The bottom row (Figure 5, *A*) showed the corresponding MAP images. As shown, ovarian tumors of the mice that received injection of NIR-830-Z_{HER2:342}-IONP were clearly imageable even after the addition of a lay of 19 mm exogenous tissues. Signal to background ratio (SBR) decreased from 38dB to 17dB when imaging depth increased. We also investigated the imaging depth of FMT (third and fourth rows in Figure 5, *B*) and found an imaging depth of up to 10mm.

We then compared the spatial resolution of FMT and PAT imaging at different depths. Representative 3D images of PAT and FMT with different thicknesses of the chicken breast were shown in Figure S5. MAP images and typical cross-sections of both PAT and FMT was shown in Figure 5, *B*. We found that the axial and lateral resolutions of FMT were relatively poor. Although the position of image center was in the right depth, the axial and lateral sizes were much larger than the actual sizes. In contrast, high lateral and axial resolutions of PAT made the reconstructed lateral and axial sizes very close to the actual dimensions of the tumor. The recovered lateral and axial sizes of PAT and FMT were plotted (Figure 6) and the average lateral and axial full width half maximum (FWHM) of the tumor in PAT images at three different depths (3, 6 and 10 mm) were 5 and 3 mm, which are comparable to the size of the resected tumor. Using FMT, the lateral FWHM was 5 to 13mm and axial FWHM was 3mm to 14 mm, as the depth increased, which were relatively larger than the actual tumor size.

Discussion

In this report, we demonstrated the feasibility of using PAT and FMT to detect orthotropic ovarian tumors in mice after systemic delivery of HER-2/neu targeted imaging probes.

Previously, we compared the PAT imaging signals of IONPs with several other nanoparticle contrasts²¹, such as carbon nanotubes^{15, 30}, gold nanocages^{20, 31}, gold nanorods³², gold nanoshell^{33, 34}. Although our results showed that gold nanoparticles and carbon nanotubes had stronger photoacoustic contrasts than that of IONPs in animal tumor models, the effects of systemic delivery of those nanoparticles in humans have yet to be determined since the mechanisms of clearance of the nanoparticles and short- and long-term toxicity are still unclear. In contrast, IONPs are a class of biodegradable and biocompatible nanoparticles with a low toxicity in humans and have already been used in human patients for MRI detection of liver cancer and lymph node metastasis after systemic delivery.³⁵

Based on our *in vitro* test result, we found that the photoacoustic signal was generated primarily from the IONPs as the concentration of NIR-830 dye conjugated to the IONPs is too low to show a marked signal enhancement effect. However, it is feasible to enhance the PAT signal by modifying our nanoparticle design to incorporate large amount of NIR-830-dye molecules (over 1000 molecules per IONP) into the polymer-coating of the IONPs. Increased concentration of NIR dye in NIR-830-Z_{HER2:342}-IONP will further enhance the PAT signal.

Planar fluorescence imaging has been commonly used in molecular imaging for monitoring the delivery of nanoprobe. However, it lacks the ability to map tumors with the imaging probes that localize deep inside the body because of a poor spatial resolution and lack of depth information. In this study, our handheld FMT system showed the potential to noninvasively map ovarian tumors in 3D at depths up to 10mm. The imaging procedure is fast, taking less than 2 minutes to cover a 20×20mm² imaging area. Additionally, FMT can specifically detect tumors with a high sensitivity because of inherent characteristic of fluorescent imaging modality.²³ However, relatively low axial and lateral resolutions affect the imaging accuracy, which may limit future applications in human patients. As a complementary method to FMT, PAT was employed to detect ovarian tumors with contrast enhancements from IONPs. We anticipate that, in a clinical setting, the fast hand-held FMT system could be used to mark the possibly tumor containing areas, and then PAT could be used to image the marked area to accurately identify tumor margins and disseminated small tumor lesions in the peritoneal cavity. This combination imaging approach takes the advantage of both FMT and PAT to complement each single modality.

Although we have demonstrated the feasibility of targeted FMT and PAT imaging of ovarian cancers, we also recognize that further development and optimizations will be needed to overcome limitations of the imaging approaches. For example, current slow and bulky photoacoustic imaging system is not suitable for clinical applications, especially intraoperative imaging. However, this should not be a fundamental problem of PAT since the problem can be solved by using a commercial ultrasound array and/or a high frame rate of laser pulses with a high-speed scanning system.³⁶ Our hand-held FMT system is a fast and portable imaging device that can be translated into an intraoperative imaging device. Since it has the ability to detect tumors located 10 mm beneath the normal tissue, it should have sufficient imaging depth and sensitivity for intraoperative tumor imaging. To improve the depth and accuracy of noninvasive localization of small tumors using the hand-held FMT, we are currently developing new hand-held FMT devices using high compact optical fiber bundles that contain more sources and detectors. Results of simulation and tissue-mimicking phantom experiments showed that the improved FMT system can image targets located 25 mm beneath the surface. Additionally, the current mechanical scan of the light sources is time consuming. In the future, we plan to use either laser diode array or fast optical switch to shorten the total scan time to less than 5 seconds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Hoskins WJ. Prospective on ovarian cancer: why prevent? *J Cell Biochem Suppl.* 1995; 23:189–199. [PubMed: 8747396]
2. American Cancer Society. Cancer facts & figures. American Cancer Society; Atlanta, GA: 2011.
3. Ovarian cancer: screening, treatment, and followup. NIH Consensus Statement. 1994; 12(3):1–30.
4. Tammela J, Lele S. New modalities in detection of recurrent ovarian cancer. *Current Opinion in Obstetrics and Gynecology.* 2004; 16(1):5–9. [PubMed: 15128001]
5. Shaaban A, Rezvani M. Ovarian cancer: detection and radiologic staging. *Clinical Obstetrics and Gynecology.* 2009; 52(1):73–93. [PubMed: 19179862]
6. Pan HS, Lee SL, Huang LW, Chen YK. Combined positron emission tomography and tumor markers for detecting recurrent ovarian cancer. *Archives of Gynecology and Obstetrics.* 2011; 283(2):335–341. [PubMed: 20221620]
7. von Schulthess GK, Steinert HC, Hany TF. Integrated PET/CT: current applications and future directions. *Radiology.* 2006; 238:405–422. [PubMed: 16436809]
8. Mullan BP, O'Connor MK, Hung JC. Single photon emission computed tomography. *Neuroimaging Clin N Am.* 1995; 5(4):647–673. [PubMed: 8564288]
9. Beard P. Biomedical photoacoustic imaging. *Interface Focus.* 2011; 1:602–31. [PubMed: 22866233]
10. Oladipupo S, Hu S, Santeford A, Yao J, Kovalski J, Shohet R, et al. Conditional HIF-1 induction produces multistage neovascularization with stage-specific sensitivity to VEGFR inhibitors and myeloid cell independence. *Blood.* 2011; 117:4142–4153. [PubMed: 21307392]
11. Manohar S, Kharine A, van Hespén JCG, Steenbergen W, van Leeuwen TG. Photoacoustic mammography laboratory prototype: Imaging of breast tissue phantoms. *J Biomed Opt.* 2004; 9:1172–1181. [PubMed: 15568937]
12. Kruger R, Lam R, Reinecke D, Del Rio S, Doyle R. Photoacoustic angiography of the breast. *Med Phys.* 2011; 37:6096–6100. [PubMed: 21158321]
13. Xi L, Li X, Yao L, Jiang H. Design and evaluation of a hybrid photoacoustic tomography and diffuse optical tomography for breast cancer detection. *Med Phys.* 2012; 39(5):2584–2594. [PubMed: 22559629]
14. Xi L, Grobmyer S, Wu L, Chen R, Zhou G, Gutwein L, et al. Evaluation of breast tumor margins in vivo with intraoperative photoacoustic imaging. *Opt Express.* 2012; 20:8726–8731. [PubMed: 22513583]
15. Zerda ADL, Zavaleta C, Keren S, Vaithilingam S, Bodapati S, Liu Z, et al. Carbon nanotubes as photoacoustic molecular imaging agents in living mice. *Nat Nanotech.* 2008; 3:557–62.
16. Pan D, Pramanik M, Senpan A, Allen JS, Zhang H, Wickline SA, et al. Molecular photoacoustic imaging of angiogenesis with integrin targeted gold nanobeacons. *The Federation of American Societies for Experimental Biology (FASEB) Journal.* 2011; 25(3):875–82.
17. Kim C, Cho EC, Chen J, Song KH, Au L, Favazza C, et al. In vivo molecular photoacoustic tomography of melanomas targeted by bioconjugated gold nanocages. *ACS nano.* 2010; 4:4559–64. [PubMed: 20731439]
18. Pan D, Pramanik M, Senpan A, Ghosh S, Wickline SA, Wang LV, Lanza GM. Near infrared photoacoustic detection of sentinel lymph nodes with gold nanobeacons. *Biomaterials.* 2010; 31:4088–4093. [PubMed: 20172607]
19. Kim C, Song KH, Gao F, Wang LV. Sentinel lymph nodes and lymphatic vessels: noninvasive dual-modality in vivo mapping by using indocyanine green in rats-volumetric spectroscopic photoacoustic imaging and planar fluorescence imaging. *Radiology.* 2010; 255:442–50. [PubMed: 20413757]
20. Yang XM, Skrabalak SE, Li Z, Xia Y, Wang LV. Photoacoustic tomography of a rat cerebral cortex in vivo with Au nanocages as an optical contrast agent. *Nano Letters.* 2007; 7 (12):3798–802. [PubMed: 18020475]

21. Xi L, Grobmyer SR, Zhou G, Qian W, Yang L, Jiang H. Molecular photoacoustic tomography of breast cancer using receptor targeted magnetic iron oxide nanoparticles as contrast agents. *J Biophotonics*. 2012;10(2):201200155.
22. Galanzha EI, Shashkov EV, Kelly T, Kim JW, Yang L, Zharov VP. In vivo magnetic enrichment and multiplex photoacoustic detection of circulating tumor cells. *Nat Nanotech*. 2009; 4:855–60.
23. Wang B, Zhao Q, Barkey NM, Mouse DL, Jiang H. Photoacoustic tomography and fluorescence molecular tomography: A comparative study based on indocyanine green. *Med Phys*. 2012; 39(5): 2512–2517. [PubMed: 22559621]
24. Ntziachristos V. Fluorescence molecular imaging. *Annual Review of Biomedical Engineering*. 2006; 8:1–33.
25. Tan Y, Jiang H. Diffuse optical tomography guided quantitative fluorescence molecular tomography. *Appl Opt*. 2008; 47(12):2011–2016. [PubMed: 18425173]
26. Satpathy M, Wang L, Zielinski R, Qian W, Lipowska M, Capala J, Lee GY, Xu H, Wang A, Mao H, Yang L. Active targeting using HER-2-affibody-conjugated nanoparticles enabled sensitive and specific imaging of orthotopic HER-2 positive ovarian tumors. *Small*. 2013;10(2):201301593.
27. Lee SB, Hassan M, Fisher R, Chertov O, Chernomordik V, Kramer-Marek G, et al. Affibody molecules for in vivo characterization of HER2-positive tumors by near-infrared imaging. *Clin Cancer Res*. 2008; 14:3840–3849. [PubMed: 18559604]
28. Yang L, Peng XH, Wang A, Wang X, Cao Z, Ni C, Karna P, Zhang X, Wood WC, Gao X, Nie S, Mao H. Receptor-targeted nanoparticles for in vivo imaging of breast cancer. *Clin Cancer Res*. 2009; 15:4722–4732. [PubMed: 19584158]
29. Zhao Q, Jiang H, Cao Z, Yang L, Mao H, Lipowska M. A handheld fluorescence molecular tomography system for intraoperative optical imaging of tumor margins. *Med Phys*. 2011; 38(11): 5873–5878. [PubMed: 22047351]
30. Kim J, Galanzha EI, Shashkov E, Moon HM, Zharov VP. Golden carbon nanotubes as multimodal photoacoustic and photothermal high-contrast molecular agents. *Nat Nanotech*. 2009; 4:688–694.
31. Li W, Brown PK, Wang LV, Xia Y. Gold nanocages as contrast agents for photoacoustic imaging. *Contrast Media Mol Imaging*. 2011; 6(5):370–377. [PubMed: 22025337]
32. Manohar S, Ungureanu C, Van Leeuwen TG. Gold nanorods as molecular contrast agents in photoacoustic imaging: the promises and the caveats. *Contrast Media Mol Imaging*. 2011; 6(5): 389–400. [PubMed: 22025339]
33. Xiang L, Xing D, Gu H, Yang D, Zeng L, Yang S. Cold nanoshell-based photoacoustic imaging application in biomedicine. *International Symposium on Biophotonics, Nanophotonics and Metamaterials*. 2006:76–79.
34. Jain PK, Lee KS, El-Sayed IH, El-Sayed MA. Calculated absorption and scattering properties of gold nanoparticles of different size, shape, and composition: applications in biological imaging and biomedicine. *J Phys Chem B*. 2006; 110:7238–7248. [PubMed: 16599493]
35. Harisinghani MG, Barentsz J, Hahn PF, Deserno WM, Tabatabaei S, van de Kaa CH, de la Rosette J, Weissleder R. Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. *N Engl J Med*. 2003; 348(25):2491–2499. [PubMed: 12815134]
36. Erpelding TN, Kim C, Pramanik M, Jankovic L, Maslov K, Guo Z, et al. Sentinel Lymph Nodes in the Rat: Noninvasive Photoacoustic and US Imaging with a Clinical US System. *Radiology*. 2010; 256(1):102–110. [PubMed: 20574088]

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Figure 1.

Schematic and characteristics of imaging probes. (A) Excitation and emission spectrum of NIR-830 dye. Excitation wavelength: 797 nm; Emission wavelength: 815 nm. (B) Schematic representation of NIR-830-ZHER2:342-IONP: NIR-830 dye was conjugated to a cysteine residue at the C-terminal end of ZHER2:342. The ZHER2:342-NIR-830 complex was conjugated to the carboxyl groups of amphiphilic polymer-coated IONPs (21.6 nm in diameter). (C) Specific binding and uptake efficiency of the NIR-830-ZHER2:342-IONP, IONP and NIR-830-BSA-IONP. HER-2 positive SKOV3 cells treated with NIR-830-ZHER2:342-IONP, IONP and NIR-830-BSA-IONP were scraped separately and scanned for absorbance from 500nm to 784nm. Since the cells were stained with nuclear fast red, the peak absorbance for Prussian blue was seen at 528 nm. Absorbance of cells treated with NIR-830-ZHER2:342-IONP and NIR-830-BSA-IONP at 528 nm was 0.549 and 0.155. (D) Absorbance spectrum of IONP and NIR-830-ZHER2:342-IONP within NIR range. (E) Photoacoustic evaluation of NIR-830 and NIR-830-ZHER2:342-IONP with tumors without any contrast agents. The equivalent concentrations of NIR-830 and NIR-830-ZHER2:342-IONP to normal ovarian tumor were 4 picomole/μL and 0.1 picomole/μL (F) Photoacoustic intensity from different concentrations of NIR-830-ZHER2:342-IONP.

Figure 2.

In vivo fluorescence and photoacoustic imaging of tumor-bearing mice and quantitative analysis. (A) The mean fluorescence intensity plot of tumors in targeted or non-targeted group 24 hours and 48 hours post injection. Fold increases shown in the bar figure were the mean fluorescence intensity of the tumor area divided by the mean fluorescence intensity of the body background. Results were from four mice in each group. Student's t-test was used to determine the p value. (B) Bioluminescence imaging of the mice showed the position of the tumor which was used to select the imaging areas of FMT and PAT. (C) Bioluminescence images of typical mice from targeted (left), non-targeted (middle) and control (right) animal groups. (D) Planar fluorescence imaging of mice from targeted, non-targeted, and control groups 24 hours post injection of nanoparticles. (E, F) MAP images and typical cross-sections of PAT and FMT of mice in the targeted (left), non-targeted (middle) and non-injection (right) animal groups. Tumors of the mice from the targeted group were clearly detected by both PAT and FMT, while the imaging contrasts of the tumors of the mice from non-targeted and non-injection groups were too low to be detected. (G) Quantitative photoacoustic intensity plots of tumors from the targeted (red), non-targeted (black) and non-injection (blue) animal group at 730 nm, 800 nm and 870 nm.

Figure 3.

Ex-vivo evaluation of tumors and normal organs using planar fluorescence imaging, PAT and FMT. Planar fluorescence imaging of the tumor, heart, spleen, kidney and liver of the mouse in the targeted (A) and non-targeted (B) groups. PAT and FMT evaluation of the tumor and small samples of normal organs of the mice in targeted animal group (C) and non-targeted group (D).

Figure 4.

Histological and quantitative analysis of tumors and normal organs. (A) Prussian blue staining of tissue sections showed high levels of iron positive cells in the tumor of mice in targeted group and low to intermediate levels of iron positive cells in the livers and spleens of mice in both targeted and non-targeted groups. No distinct iron positive cells were visible in the tumors of mice in non-targeted group, and in the kidney and heart. PB: Prussian blue, HE: hematoxylin and eosin (B) Comparison of photoacoustic intensity of the tumors and normal organs resected from the mice in targeted and non-targeted groups. The heart and targeted tumor produced the strongest photoacoustic emission. The spleen generated intermediate photoacoustic emission.

Figure 5.

Evaluation of imaging depth and spatial resolution of PAT and FMT. (A) B-scan (top row) and MAP (bottom row) PA images of a tumor in a representative mouse that received NIR-830- $Z_{\text{HER2:342}}$ -IONP. The back flank of the mouse was overlaid with 0 mm, 10 mm and 19 mm thick layers of chicken breast to mimic the normal tissue in humans. Red arrows showed the surfaces of the chicken breast. Scale bar: 5 mm. (B) Dual modality imaging of the same tumor located at different depth (3 mm, 6 mm and 10 mm). The dashed white lines indicated the position of the selected cross-sections of PAT and FMT and the red arrows showed the positions of the imaging surfaces. Scale bar: 5 mm. From the MAP and cross-sections of PAT and FMT, we noticed that the lateral and axial resolutions of PAT were higher than that of FMT.

Figure 6.

Quantitative comparison of spatial resolutions between PAT and FMT with increased imaging depth. Lateral profiles of the recovered tumor by PAT (A) and FMT (B) along the dashed white lines in Figure 5 B. Axial profiles of the recovered tumor by PAT (C) and FMT (D).