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Optical Imaging of Ovarian Cancer Using HER-2 Affibody Conjugated Nanoparticles

Minati Satpathy, Rafal Zielinski, Ilya Lyakhov, and Lily Yang

Abstract

Computed Tomography (CT), Ultrasound (US), and Magnetic Resonance Imaging (MRI) have been the mainstay of clinical imaging regimens for the detection of ovarian cancer. However, without tumor specific contrast enhancement, these imaging modalities lack specificity and sensitivity in the detection of small primary and disseminated tumors in the peritoneal cavity. Herein, we illustrate a fairly new near infrared (NIR) optical imaging approach developed in our laboratory for the noninvasive detection of ovarian tumors using a HER-2 targeted nanoparticle-based imaging agent in an orthotopic mouse model of ovarian cancer. We used multimodal imaging approaches to detect the disease accurately and rapidly by utilizing a single imaging agent, NIR dye-labeled HER-2 affibody conjugated iron oxide nanoparticles. This agent targets HER-2 receptors, which are overexpressed in ovarian tumors. This chapter outlines materials and methods for the: (1) production of HER-2 targeted nanoparticles; (2) establishment of an orthotopic human ovarian cancer xenograft model; (3) monitoring of tumor growth by bioluminescence imaging; (4) administration of targeted nanoparticles followed by NIR optical imaging for the detection of orthotopic ovarian cancers with targeted accumulation of the nanoparticle imaging probes.

Keywords

Near infrared optical imaging; Iron oxide nanoparticles; HER-2 affibody; Orthotopic model of ovarian cancer; Bioluminescence imaging

1 Introduction

Peritoneal metastases are the major contributing factor for the increased mortality rate among ovarian cancer patients. Early diagnosis and accurate staging are the key determinants for the successful treatment of this disease. Recent advancement in nanotechnology and molecular imaging bring a ray of hope for the patients with advanced ovarian cancer. These technologies are emerging as powerful tools for the precise detection and identification of many cancers by using biomarker targeted contrast agents. Targeted imaging and personalized therapy should have a significant impact on overall management of ovarian cancer patients [1]. One such bio-marker, HER-2/*neu*, is known to be up-regulated in about 25 % of ovarian cancer patients and is correlated with poor survival [2]. HER-2 positive tumors have aggressive tumor biology and are resistant to chemotherapy and molecular targeted therapy, such as monoclonal antibody against HER-2 or Herceptin [3]. Surgical resection of ovarian tumors is a common treatment for ovarian cancer. Current methods for identification of an ovarian cancer lesion in the peritoneal cavity rely on the

gross appearance of the tumor tissues, which lacks specificity and sensitivity. The development of tumor targeted imaging agents and imaging methods will provide a useful means for sensitive detection of ovarian tumors by preoperative noninvasive imaging and intraoperative image-guided surgery.

Recently, a novel affinity molecule, HER-2 affibody, has been developed that is small in size, highly stable, and engineered with a unique cysteine residue at the C terminus end for site specific labeling of imaging contrast agents [4]. More importantly, HER-2 affibody ($Z_{HER2:342}$) specifically binds to HER-2, a different epitope than Herceptin [5], which makes it suitable for patients subjected to Herceptin treatment.

Near infrared (NIR) fluorescence imaging is an emerging imaging approach receiving significant attention as a potential means to detect cancer by noninvasive or intraoperative optical imaging [6, 7]. This method is accomplished by labeling an optical contrast agent (e.g., NIR dye) to a targeting ligand that binds to a target molecule highly expressed in tumor cells or tumor microenvironment. Systemic delivery of targeted imaging probes leads to the accumulation of the probes at the tumor site due to the molecular recognition of the tumor biomarker. At present, optical imaging using nontargeted NIR dyes, such as indocyanine green (ICG), has been used in patients for various clinical applications [8]. A recent study revealed the detection of ovarian tumors in human patients by using fluorescein isothiocyanate-labeled folic acid probes that target to folate receptor highly expressed in ovarian cancers [9]. A number of other preclinical studies has used NIR optical imaging probe for in vivo tumor imaging [7, 10]. Currently the Food and Drug Administration (FDA) has approved two NIR fluorophore dyes: ICG and methylene blue (MB). However, a number of research groups are developing novel NIR molecular imaging agents for optical imaging of tumors.

Magnetic iron oxide nanoparticles (IONPs) are potential multi-functional clinical tools for targeted imaging and drug delivery [11]. The iron oxide nanoparticle is biocompatible and biodegradable with low toxicity. Clinically nontargeted IONPs have been used as a contrast agent for magnetic resonance imaging in the detection of lymph nodes [12]. We have conjugated a NIR dye-labeled HER-2 affibody to amphiphilic polymer coated IONPs to produce a dual optical and MR imaging probe, NIR-830- $Z_{HER-2:342}$ -IONPs [13]. This multifunctional nanoprobe is capable of targeting HER-2 over-expressing ovarian tumors that is detectable by multimodal imaging methods such as NIR optical imaging, magnetic resonance imaging (MRI), photoacoustic tomography (PAT), fluorescence molecular tomography (FMT), and spectroscopic imaging technique in an orthotopic ovarian tumor mouse model [14].

This chapter outlines our methods of (a) production of recombinant HER-2 affibody; (b) preparation of NIR dye-labeled HER-2 affibody conjugated IONPs; (c) generation of an orthotopic mouse model for ovarian cancer; (d) detection of ovarian tumor progression by bioluminescence imaging (BLI); and (e) detection of orthotopic ovarian cancer by NIR optical imaging and related image processing techniques.

2 Materials

2.1 Production of Recombinant HER-2 Affibody

1. HER-2 affibody (obtained from Affibody AB, Stockholm, Sweden on Collaborative Research and Development agreement); this protocol can also be used with other antibodies but the disulfide bonds in the protein has to be broken by appropriate reducing agent before labeling with the NIR amine ester dye.
2. Super Broth (Quality Biological, Gaithersburg, MD); LB broth can also be used but yield may be lower.
3. Ampicillin.
4. Isopropyl β -D-1-thiogalactopyranoside (IPTG).
5. Imidazole.
6. Protease Inhibitor Cocktail.
7. Dithiothreitol (DTT).
8. Tobacco Etch Virus (TEV) protease.
9. HIS TRAP column.
10. Protein purification and concentration filters (i.e., Amicon Ultra Sample concentration unit, EMD Millipore).
11. Modular liquid chromatography system (i.e., AktaPurifier10, GE Healthcare).
12. Centrifuge.
13. Sonic Dismembrator.
14. Temperature-controlled benchtop orbital shaker.

2.2 Preparation of Near Infrared Labeled HER-2 Affibody Conjugated Iron Oxide Nanoparticles

1. Iron oxide nanoparticles (core size 10 nm) coated with amphiphilic polymers (we use particles from Ocean NanoTech, LLC, Springdale, AR).
2. Maleimide dye (we use NIR-830 maleimide dye synthesized from IR-783 dye (Sigma-Aldrich)) using a synthesizing method as described in [15] but any commercially available can be used following this protocol.
3. Borate buffer pH 5.5 and 8.6.
4. Tris[2-carboxyethyl] phosphine (TCEP).
5. Ethyl-3-dimethyl amino propyl-carbodiimide (EDAC).
6. Sulfo-*N*-hydroxysuccinimide (sulfo-NHS).
7. Ultrafiltration system (i.e., Nanosep 100 K column, Pall Corporation, Ann Arbor, MI).

8. Microfuge tubes.
9. Lab shaker.
10. Spectrophotometer.
11. Dynamic light scattering (DLS) instrument (i.e., Zetasizer Nano-ZS S-90, Malvern Instruments Inc., Southborough, MA).

2.3 Establishment of Orthotropic Human Ovarian Cancer Xenograft Model

1. 6–8 week old female athymic nude mice.
2. SKOV3 ovarian cancer cell line stably transfected with luciferase gene (SKOV3-luc) (kindly provided by Dr. Daniela Matei at Indiana University Purdue University at Indianapolis); any other cancer cell line overexpressing the antigen of interest (in our case HER-2) may be used and transplanted orthotopically as long as they express the luciferase reporter.
3. McCoy's 5A culture medium with 10 % fetal bovine serum or appropriate culture medium.
4. Trypsin.
5. 1× PBS.
6. D-luciferin.
7. Tissue culture incubator maintained at 37 °C with 5 % CO₂.
8. Water bath adjusted to 37 °C.
9. 15 ml conical tubes.
10. Hemocytometer.
11. 0.5 ml insulin syringe.
12. Ketamine hydrochloride and xylazine hydrochloride in sterile saline.
13. Autoclaved surgical instruments (scissors, forceps, surgical wound staples, ear punch, polypropylene suture 18").
14. Bio shield sterile wrap super, sterile surgical sponges, surgical gloves, and mask.
15. Alcohol Prep pad and Povidone-Iodine prep pad.
16. Heating pad.
17. Digital camera.
18. Meloxicam (Metacam stock 5 mg/ml).
19. Mouse restrainer.

2.4 Preclinical In Vivo Imaging Systems

IVIS in vivo imaging system (Xenogen Corporation, Caliper Life Sciences).

Kodak in vivo imaging system Fx (Carestream Health Inc., New Haven, CT).

3 Methods

Production of recombinant HER-2 Affibody molecule ($Z_{\text{HER2:342}}\text{-(GS)7-Cys}$) (*see* Note 1) involves: (a) overexpression and purification of MBP-TEV-6His- $Z_{\text{HER2:342}}\text{-(GS)7-Cys}$ construct (*see* Note 2); (b) proteolytic removal of MBP tag; and (c) purification of the final product. The plasmid pMTZC for expression of HER-2 Affibody molecules ($Z_{\text{HER2:342}}\text{-(GS)7-Cys}$ or $Z_{\text{HER2:342}}\text{-GSC}$) was cloned as described in [16–18]. The yield of the protocol described below is approximately 4 mg/l.

3.1 Overexpression of MBP-TEV-6His- $Z_{\text{HER2:342}}\text{-(GS)7-Cys}$

1. Inoculate 100 ml of LB broth medium supplemented with ampicillin at 100 µg/ml with frozen stock of *E. coli* BL21(DE3) transformed with pMTZC plasmid. Grow bacterial culture O/N with intensive shaking at 37 °C.
2. Measure the OD₆₀₀ of the O/N culture and inoculate 1 l of LB broth medium supplemented with ampicillin at 100 µg/ml. The initial OD₆₀₀ should be 0.1–0.15 AU. Grow bacteria at 37 °C with intense shaking 180–220 rpm, periodically checking OD₆₀₀.
3. Once bacteria have reached the density 1.0 AU, induce protein expression by adding IPTG to final concentration 1 mM, continue bacterial culture for 3 h.
4. Centrifuge bacteria 4,000 × g, 10 min, resuspend in 100 ml ice-cold PBS supplemented with complete protease inhibitor cocktail tablets, and sonicate in ice-bath 2 × 5 min using Sonic Dismembrator at 20 W.
5. Remove cell debris by centrifugation 48,000 × g, 20 min and filter the supernatant through 0.22 µm filter.

3.2 Purification and Proteolytic Removal of MBP Protein

1. Equilibrate HisTrap (5 ml) column with at least 20 volumes of ice-cold 50 mM phosphate buffer containing 300 mM NaCl and 10 mM imidazole, pH 8.
2. Load the supernatant onto the column at 0.5 ml/min using loading superloop (GE HealthCare).
3. Washout unbound proteins by running through the column ice-cold 50 mM phosphate buffer containing 300 mM NaCl and 10 mM imidazole until UV monitor returns to baseline level.
4. Elute resin-bound protein using linear gradient of imidazole from 0 to 300 mM in 50 mM phosphate buffer with 300 mM NaCl, pH 8 over 10× column volumes. Collect eluate in 0.5 ml fractions. Analyze fraction by SDS/PAGE.

¹(GS)7-flexible linker is added to HER-2 affibody to lower the probability of steric interaction of the label attached to the C-terminal cysteine with the binding site.

²Maltose binding protein (MBP) is used to increase the stability and yield of $Z_{\text{HER2:342}}\text{-GSC}$.

5. Based on electropherogram, combine fractions containing MBP-TEV-6His-Z_{HER2:342}-GSC (expected molecular weight approximately 50 kDa). Exchange imidazole-containing buffer to PBS, using Amicon Ultracentrifugal filter units (30 kDa, cut off).
6. Add DTT to final concentration 1 mM followed by incubation with TEV protease at 0.1 mg/ml overnight at room temperature. Dilute the sample to 1 mg/ml of protein in PBS.
7. Repeat purification on HisTrap columns (**steps 1–6**), collect fractions based on electrophoretic migration (expect the band of approx. 10 kDa in reducing SDS/PAGE condition). Dilute protein to 1 mg/ml, pass through 0.22 µm filters using sterile PBS, aliquot in 0.5 ml. Store at –80 °C.

3.3 Preparation of NIR-830-HER-2 Affibody-IONPs

1. Prepare fresh 0.5 M TCEP in 1× PBS.
2. Add 1 µl of TCEP (stock 0.5 M) to 150 µg Z_{HER2:342}-GSC (hereafter referred to as Z_{HER2:342}) for a total volume of 100 µl in PBS, pH 7.4.
3. Incubate the mixture at room temperature for 15–30 min.
4. Prepare a 10 mM stock of NIR-830 maleimide (mol wt. 965.327) in DMSO.
5. Add fourfold molar excess of NIR-830 maleimide dye or commercially available NIR maleimide dyes over the concentration of Z_{HER2:342} directly to the mixture and incubate the mixture in a sealed tube at 4 °C overnight with rotation (*see* Note 3).
6. Take 400 µl of amphiphilic polymer coated iron oxide nanoparticles (Stock 5 mg/ml), activate with 200 M of EDAC and 100 M of sulfo-NHS in 10 mM Borate buffer at pH 5.5 according to the carbodiimide method, and then allow it to rotate for 15 min at room temperature (*see* Note 4).
7. Immediately after incubation, neutralize the reaction by adding 10 mM Borate buffer, pH 8.6, and wash the mixture by using a Nanosep 100 K spin column.
8. Add the bi-conjugate NIR-830-Z_{HER2:342} to the activated IONP (at a molar ratio Z_{HER2:342}-IONP10:1) and allow it to incubate for 4 h at room temperature with rotation (*see* Note 5).
9. Purify the final product NIR-830-Z_{HER2:342}-IONP (Fig. 1a) with 10 mM Borate buffer, pH 8.6, using Nanosep 100 K spin column to remove the reaction by-products.

³HER-2 affibody contains a disulfide bond which needs to be reduced before labeling with a maleimide. TCEP reduces the disulfide bond effectively as compared to DTT. Since it does not contain thiols, it does not have to be removed from the solution before adding maleimide reaction chemistry.

⁴EDAC is used as a catalyst to improve the reaction activity of sulfo-NHS with COOH functional groups on the polymer coating of the nanoparticles. Conjugation is prepared via cross-linking of carboxyl group of the amphiphilic polymer coating to the amino side groups of Z_{HER2:342} affibody.

⁵HER-2 affibody is small in size and removal of the reducing agent after the reduction step may cause loss of some HER-2 affibody resulting in poor conjugation, so avoid washing the bi-conjugate (NIR-830-Z_{HER2:342}) at this step.

10. Resuspend in 10 mM Borate buffer, pH 8.6, and store at 4 °C.
11. NIR-830-Z_{HER2:342}-IONP generated by using this method is stable for at least 6 months at 4 °C.
12. Measure the concentration of iron in the resulting conjugates by taking the absorbance at O.D. 500 nm (*see* Note 6).
13. Measure the hydrodynamic size of NIR-830-Z_{HER2:342}-IONP by using the dynamic light scattering (DLS) instrument by preparing a 1 mg/ml solution in water followed by a quick sonication (Fig. 1b).

3.4 Establishment of Orthotopic Human Ovarian Cancer Xenograft Model

1. Culture SKOV3-luc ovarian cancer cells in McCoy 5A medium supplemented with 10 % fetal bovine serum and 1 % penicillin and streptomycin, and then allow them to grow at 37 °C, 5 % CO₂.
2. When cells reach 80 % confluence, trypsinize and wash cells twice with culture medium at 200 × g for 5 min followed by a final PBS wash. Count cells using a hemocytometer (*see* Note 7).
3. Resuspend cells in PBS and transfer 6 × 10⁴ cells in 200 µl PBS to each microfuge tube depending on the number of mice to be injected. Keep extra cells in tubes in case of loss or improper injection.
4. Spin down the cell suspension at 200 × g for 5 min and remove the supernatant carefully without dislodging the cells by using a pipette (*critical step*) leaving 15–20 µl PBS in the tube and immediately place the tube with cells on ice in preparation for orthotopic injection.
5. Each mouse will be injected orthotopically with 15–20 µl PBS containing at least 5 × 10⁴ cells into the ovarian bursa (*see* Note 8).
6. Anesthetize the mice by injecting a mixture of 95 mg/kg ketamine hydrochloride and 5 mg/kg xylazine of body weight in sterile saline intramuscularly.
7. Place the mouse on sterile pad dorsally and disinfect the operating skin area with alternate povidone-iodine pad swab followed by an alcohol pad (three times).
8. Make an abdominal incision using a sharp scissor and forceps to expose the right ovary. Carefully look for fallopian tube using forceps to locate the ovarian bursa on the top of the fallopian tube. Gently lift the ovarian bursa along with the fat pad and inject 20 µl of the cell suspension using a 0.5 ml insulin syringe attached to a 27G needle into the bursa area (Fig. 2a–c, f) (*see* Note 9).

⁶Dilute the final products NIR830-Z_{HER2:342}-IONP, 50–100 times in water or PBS buffer then take the OD at 500 nm. Multiply the absorbance value with the dilution factor then divide by a factor of 4.3 to get the iron concentration in mg/ml.

⁷Calculate the total number of cells to be used for the animal experiments, pipette out calculated volume of cell suspension, then divide by number of animals to be injected in different microfuge tubes. Include extra cells in each tube in case of cell loss during the washing steps.

⁸While putting 20 µl cell suspensions using P-200 pipette by removing the plunger of a 0.5 ml insulin syringe, make sure no bubbles are being introduced.

⁹Close attention should be paid to avoid any spillage of cell suspension outside the bursa.

9. Close the abdominal incision in two separate layers. First close the peritoneal muscle followed by the outer skin using suture (18") (Fig. 2d–e). Punch the ear or use a number tag to identify individual mice for each experiment.
10. Move the mouse back to the cage and place the cage on the heating pad until the mouse is completely awake.
11. Administer pain medication, Meloxicam (metacam), 2 mg/kg immediately after surgery and then 24 and 48 h after the operation (as needed), examine the mice daily and monitor the tumor growth noninvasively by bioluminescence imaging (BLI) weekly.

3.5 In Vivo Bioluminescence Imaging (BLI) for Tumor Progression

1. Anesthetize the mice by injecting a mixture of 95 mg/kg ketamine hydrochloride and 5 mg/kg xylazine in sterile saline intraperitoneally. Start the BLI to monitor the tumor growth on week 2 after implanting the SKOV3-luc cells into the ovarian bursa (*see* Note 10).
2. Inject the luciferin substrate 30 mg/kg body weight intraperitoneally into the nude mice bearing SKOV3-luc tumor 10 min prior to imaging using a 27G, 0.5 ml insulin syringe (*see* Note 11).
3. Place the anesthetized mice inside the BLI system. Use the field of view at “B” position so that five mice can be imaged at a time.
4. Follow the instruction of the manufacturer from Caliper Life Sciences imaging system to capture the BLI. For luminescent image acquisition, use an integration time of 10 s and reduce or increase the exposure time (Fig. 3a) as per the intensity of the luminescence signal to avoid saturation. For comparative studies, capture multiple images weekly at the same exposure time (*see* Note 12).
5. For quantification of luminescence signal, select region of interest (ROI) by encircling tumor area as well as body background from displayed images using IVIS software. Calculate integrated flux of photons (total flux p/s) in each region and subtract total flux p/s value of body background from tumor intensity (total flux p/s) every week to follow the longitudinal tumor progression (Fig. 3b).
6. At week 6, when tumor is >6 mm in size, inject 400 pm of NIR-830-Z_{HER2}:₃₄₂ – IONP conjugate dissolved in PBS pH 7.4 in a total volume of 120 µl intravenously via the tail vein (Fig. 3c) of the mouse.

¹⁰SKOV3-luc cells injected orthotopically into the ovary and tumors do not project outside the skin until their sizes reach >1.0 cm. So BLI is the most sensitive regimen to monitor kinetics of the bioluminescent signals for quantification and comparison of signal during longitudinal studies for the firefly luciferase expressing cells. When injecting 5×10^4 SKOV3-luc cells into the tumor bursa, the expected size of the tumor by days 7–10 is about 1–2 mm.

¹¹Typically a dose of 150 mg/kg D-luciferin substrate is recommended. This dose can be reduced if the luciferin is freshly made and good activity of stably expressing luciferase gene in cancer cell line is observed.

¹²Since the ovary is located deep inside the abdominal cavity, capture images both dorsally and ventrally so that any spontaneous or time dependent metastases can be viewed. Our purpose is to include BLI to visualize metastases noninvasively and based on the location of metastases we can pay closure attention to those areas more carefully for the delivery of targeted nanoparticles by NIR in vivo imaging.

3.6 Near Infrared (NIR) Optical Imaging by Kodak In Vivo Imaging System Fx

1. Anesthetize the mice 24 h following the imaging probe administration by injecting a mixture of 95 mg/kg ketamine hydrochloride and 5 mg/kg xylazine body weight per mouse in sterile saline intramuscularly.
2. Inject luciferin (30 mg/kg) intraperitoneally to each mouse for noninvasive whole body bioluminescence imaging to determine tumor locations and sizes as described in Subheading 3.5.
3. Place the animal on a clear tray ventrally for NIR optical imaging. Acquire all the images with a filter set of excitation wavelength at 800 nm and emission wavelength at 830 or 850 nm, exposure time at 180 s with a gamma value of 0.2. Positioning of tumor bearing mouse is very critical for capturing the NIR images by Kodak in vivo imaging system FX (Carestream Health Inc., New Haven, CT) (*see* Notes 13 and ¹⁴). Follow other equipment settings as per the manufacturer's instructions.
4. After acquiring NIR images in each position, acquire a corresponding X-ray image to provide a clear anatomical location of the tumor (Fig. 4a).
5. ROIs containing equal pixels are drawn for tumors, kidney area, corresponding body background and tray background using accompanied Kodak in vivo imaging system Fx software package (Fig. 4b). Mean fluorescence intensities (MFI) are computed from the accompanied software. Signal-to-noise ratios (SNR) are calculated by dividing the MFI of tumor by MFI of body background. It is expected to visualize some NIR signal in the kidney within 24 h of nanoparticle delivery due to the excretion of cleaved nanoparticle products. In addition, iron oxide nanoparticles are readily taken up by kupffer cells in the liver and macrophages in the spleen which render bright NIR signals in the liver and spleen *ex vivo* but their signals were very low in noninvasive NIR imaging. When comparing kidney signal with tumor signal is needed, subtract the body background from both the tumor site and noninjected site. Finally divide the MFI of tumor by MFI of kidney site to get a fold increase MFI in the tumor region. At least three to five mice are needed to calculate the standard deviation.
6. Delivery of NIR dye-labeled HER-2 targeted nanoparticles to small metastatic ovarian tumors is difficult to be visualized by noninvasive optical imaging. Although NIR signals can be detected on the optical images, they usually lack sufficient resolution to determine size and locations of the tumor lesions. After 24

¹³To study the bio-distribution of NIR labeled targeted nanoparticles on organs, first open the abdominal muscle and remove any accumulated ascites which may interfere in NIR signal, using a 1 ml syringe or pipette. Image the ascites to determine whether the floating tumor cells can be targeted by the HER-2 targeted imaging agent. Place the mouse face down keeping all the organs intact. Use clear tape to press the mouse so that it is aligned very closely to the tray. When the primary tumor size is >6 mm, it is hard to visualize NIR signals on the metastatic small tumors. In this case, remove all the tumors, which have the highest NIR signal sequentially and adjust the gamma scale to visualize the NIR signal in any smaller tumors present in the peritoneal cavity.

¹⁴Since mouse ovary is located anatomically very close to kidney, when the tumor grows to over 6 mm in diameter, it is almost adhered to the kidney. However un-conjugated and cleaved products of NIR dye-labeled ZHER2:342 conjugated IONPs are eliminated through the kidney, which is a potential problem for NIR imaging in an ovarian orthotopic mouse model. To solve this issue, acquire the images from the dorsal side, ventral side, tumor injection site, and opposite of injection site. In addition, body background, especially in folded skin areas, may generate some artifacts in this preclinical imaging system.

and 48 h of nanoparticles delivery, capture the whole body imaging for specific accumulation of nanoparticles at the tumor site and subsequently euthanize the mice by cervical dislocation.

7. Open the skin and abdominal muscle, and place the mouse facing down on the tray to capture optical images of the exposed mouse organs. Immediately excise the tumors, normal organs and tissues for ex vivo organ imaging using NIR, BLI, and bright field imaging (*see* Note 13).
8. Keep blood and muscle samples for background correction. Primary and metastatic tumors from a representative mouse showing the targeted delivery of the nanoparticles (Fig. 4c).
9. Keep tumor and normal organ samples at -80°C for future histological analysis.
10. Figure 4d illustrates the critical positioning of mice as well as the gamma scale adjustment in the Kodak in vivo imaging system Fx (*see* Note 14).

4 Notes

All animal experiments should be performed in guidance with the protocol approved by the appropriate Institutional Animal Care and Use Committee IACUC. Current animal experiment protocol has been approved by Emory University, IACUC, Atlanta, GA, USA. Ketamine can be obtained from the pharmacy with a special order from a licensed physician.

¹(GS)7-flexible linker is added to HER-2 affibody to lower the probability of steric interaction of the label attached to the C-terminal cysteine with the binding site.

²Maltose binding protein (MBP) is used to increase the stability and yield of Z_{HER2:342}-GSC.

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Fig. 1.

- (a) Schematic illustration of optical imaging probe conjugated to iron oxide nanoparticles.
 (b) Hydrodynamic size measurement by dynamic light scattering (DLS) instrument

Fig. 2. Stepwise illustration of orthotopic implantation of SKOV3-luc cells into ovarian bursa: **(a)** Location of right and left ovaries (*blue arrowheads*). **(b)** Surgical site. **(c)** Exposed implantation site and implantation of SKOV3-luc cells into ovarian bursa. **(d)** Closing of surgical site in layers using suture. **(e)** Completely sewed. **(f)** Fallopian tube with ovarian bursa (*upper panel*) and fallopian tube with the primary ovarian tumor (*lower panel*)

Fig. 3.

(a) Representative mouse showing longitudinal tumor progression by bioluminescence imaging; (b) Analysis of luminescence signal selecting region of interest using inbuilt software; (c) Tail vein injection of NIR-830-Z_{HER2:342}-IONPs to the SKOV3-luc tumor bearing mouse

Fig. 4.

(a) Representative mouse showing target specificity of the nanoparticles at the tumor site after 24 h of nanoparticle delivery; (b) Analysis of NIR optical signal about selecting the region of interest using inbuilt Kodak in vivo imaging FX software. Analysis window also showing a table detailed about all the parameters that depend on the goal of each study; (c) Ex vivo NIR imaging of primary (#1) and metastatic tumors (#2 and #3) from a representative mouse. Corresponding bioluminescence and white field images are also included; (d) Noninvasive NIR optical imaging of targeted delivery of nanoparticles (24 h) of a representative mouse whole body showing NIR signal intensities from dorsal, ventral, tumor cells injected site and noninjected site of the mouse along with gamma scale. Bioluminescence imaging of whole body indicating the tumor location of the corresponding mouse