Electronic Supplementary Information

Dual-modal in vivo fluorescence and photoacoustic imaging using heterodimeric peptide

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Supplementary Information

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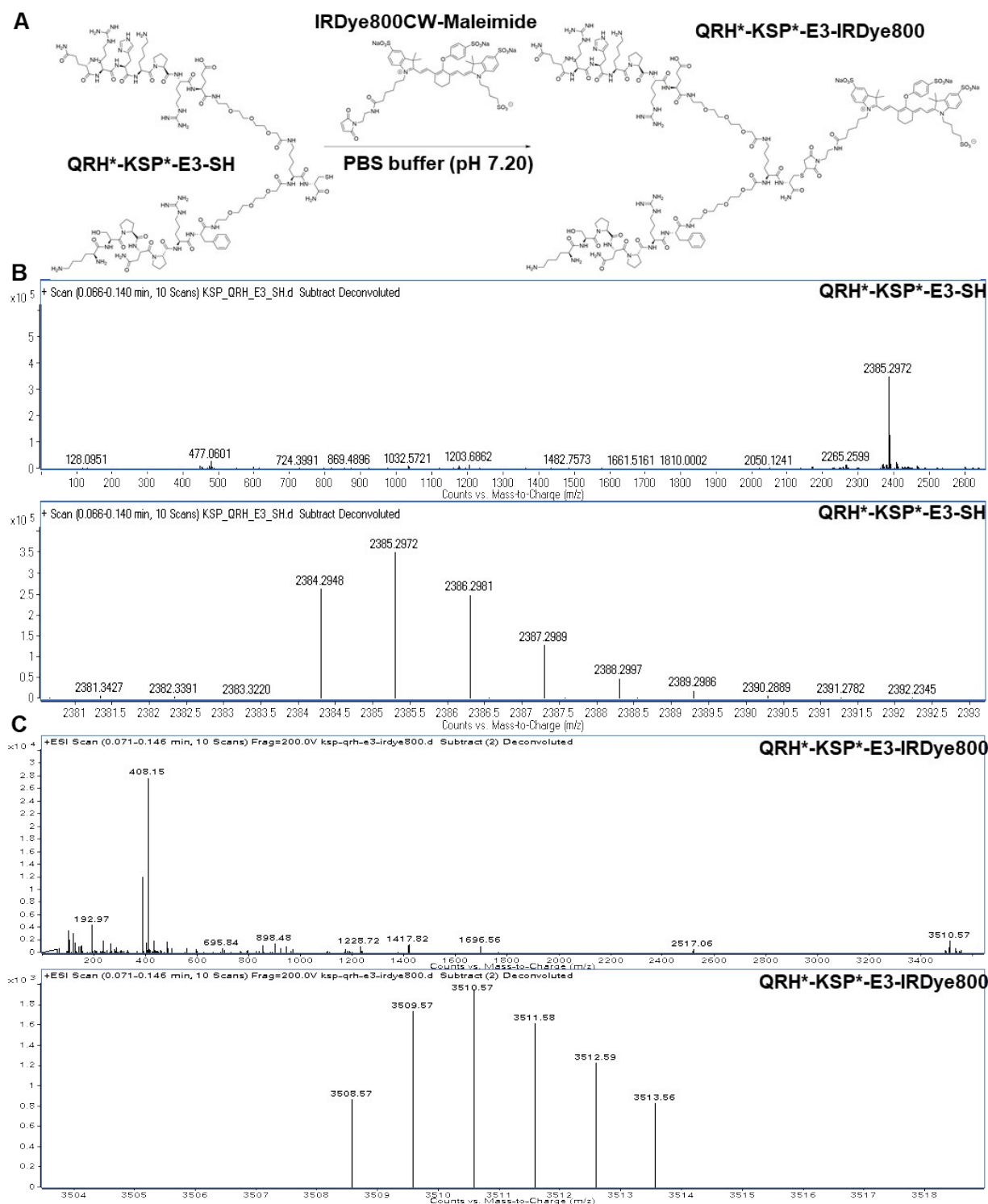
1. Supplementary Figures

Fig. **S1**. **Peptide heterodimer**. **A)** Schematic is shown for labeling of heterodimer with IRDye800. Experimental mass-to-charge (m/z) ratios for **B)** QRH*-KSP*-E3-SH and **C)** QRH*-KSP*-E3-IRDye800 were found to be 2385.29 and 3510.57, respectively, which agree with expected values.
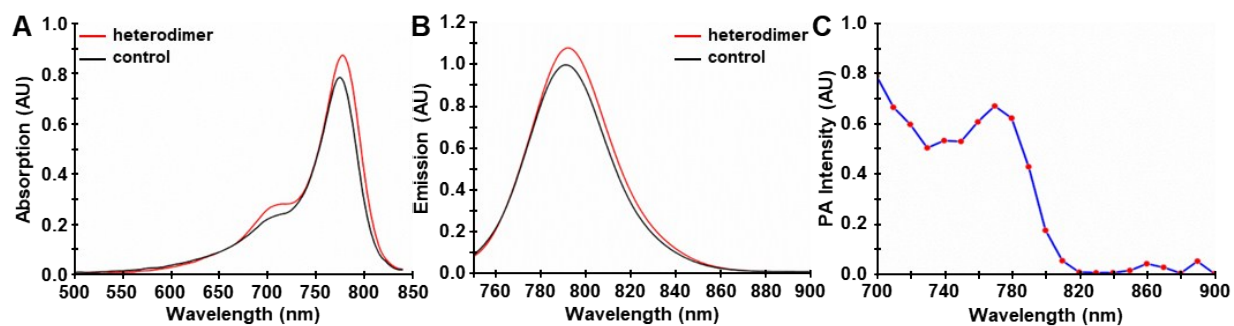
Fig. **S2**. **Spectral Characterization**. Normalized **A)** optical absorbance and **B)** fluorescence emission spectra for QRH*-KSP*-E3-IRDye800 and (GGGAGGG)$_2$-E3-IRDye800 (control) peptides show peak values at λ_{ab} = 780 and λ_{em} = 795 nm, respectively. **C)** The PA intensity shows a peak value at λ_{ab} = 770 nm.
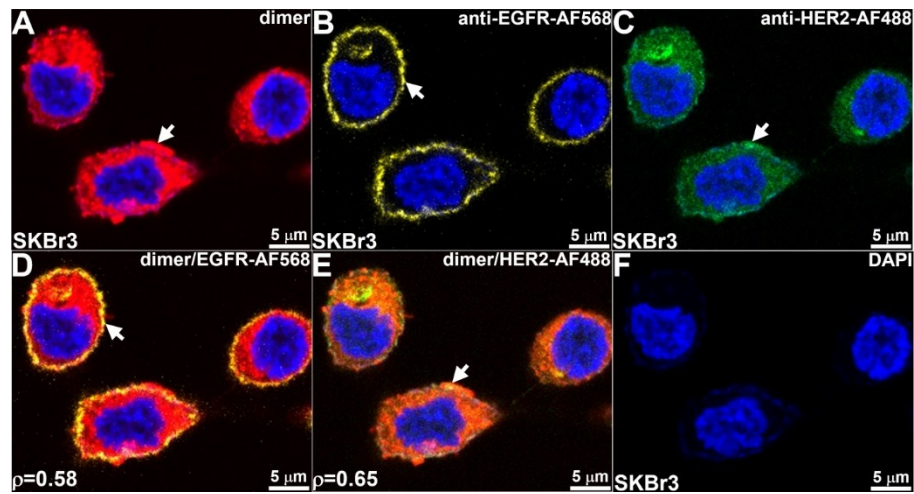
Fig. **S3**. **Co-localization of heterodimer and antibody binding**. Strong binding is seen by **A**) QRH*-KSP*-E3-IRDye800 (red), **B**) AF568-labeled anti-EGFR antibody (yellow), and **C**) AF488-labeled anti-HER2 antibody (green) to the surface (arrow) of SKBR3 cells. Merged image of heterodimer with **E**) anti-EGFR and **F**) anti-HER2 result in Pearson's correlation coefficient of ρ = 0.58 and 0.65, respectively. **F**) DAPI stain shows SKBr3 nuclei.

Fig. **S4**. **Immunohistochemistry**. Cell surface (arrow) expression of **A)** EGFR and **B)** HER2 is seen in OE33 tumor section.

2. Materials and Methods
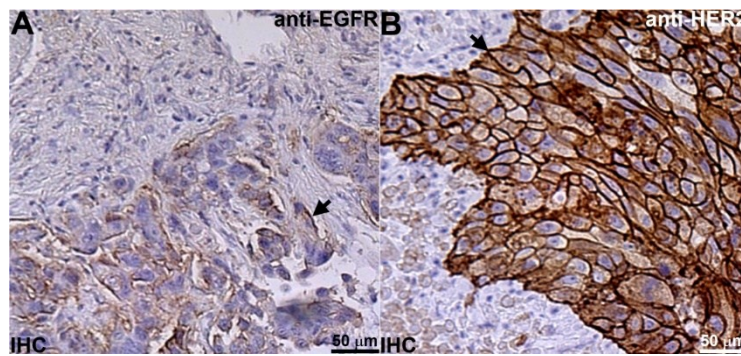
2.1 Peptide heterodimer

The heterodimeric peptide was synthesized using standard Fmoc solid-phase chemical synthesis with rink amide MBHA resin in a PS3 automatic synthesizer (Protein Technologies Inc). Fmoc protected L-amino acids were applied with standard HBTU/HOBt activation. The C-terminus lysine was incorporated as Fmoc-Lys(ivDde)-OH for fluorophore labeling. Upon completion of peptide assembly, the resin was washed with dimethylformamide (DMF) and dichloromethane (DCM) for 1 min 3X. A cleavage cocktail reagent TFA: TIS: H_2O (95:2.5:2.5 v/v/v) was mixed with the resin, and stirred for 2 hours in dark conditions at 25°C. The crude peptides were isolated from the resin by filtration and evaporated with N_2 gas followed by precipitation with chilled diethyl ether and stored at -20°C for 12 hours. The precipitated peptides were centrifuged and washed 2X with ether, dried, dissolved in water, and purified by prep-HPLC with a C18 column (Waters Inc) using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient. The purified peptide was lyophilized to produce a white powder. Labeling with IRDye800 was carried out in 1X PBS using a reducing agent tris(2-carboxyethyl)-phosphine (TCEP) at 25°C overnight. The mixture was purified by prep-HPLC, and lyophilized to produce a green appearing powder. Peptide purity was tested using an analytical C18-column. Further characterization was confirmed by either ESI (Waters Inc) or Q-TOF (Agilent Technologies) mass spectrometry.

2.2 Heterodimer properties

The absorbance spectra of the IRDye800-labeled peptides were characterized using a spectrophotometer (NanoDrop 2000, Thermo Scientific). Fluorescence excitation and emission from a 1 μM peptide solution diluted in PBS was collected with fluorimeter (Fluoromax-2) using $\lambda_{ex} = 745$ nm. The fluorescence quantum yield Φ for the IRDye800-labeled peptides is estimated

as 3.6% from the measured area-under-the-emission curve and using the published value of $\Phi=3.4\%$ for free IRDye800.[34] The spectra were plotted with Origin 8.5 software (OriginLab Corp).

A tomography system (Nexus128, Endra, Inc) was used to collect the photoacoustic images. A laser tunable between 680-950 nm provides 7 ns pulses at 20 Hz pulse (25 m□/pulse). Anesthetized animals were placed in a transparent imaging tray located in a hemispherical bowl filled with water. Acoustic signals were acquired with 128 unfocused transducers (3 mm diameter) with 5 MHz center frequency arranged in a helical pattern. System electronics perform data acquisition, image reconstruction, control of servo motors to rotate the bowl, and temperature monitoring of the water bath.

2.3 Cell culture

All cells were maintained at 37°C and 5% CO_2 and were supplemented with 10% FBS and 1% penicillin/streptomycin. Human SKBr3 breast cancer cells were obtained from the American Type Culture Collection (ATCC) and cultured in McCoy's 5A media. Human OE33 and QhTERT esophageal cells were cultured with Roswell Park Memorial Institute (RPMI) 1640 media and keratinocyte-SFM media (Gibco), respectively. Cells were passaged using 0.25% EDTA containing trypsin (Mediatech), and a hemocytometer was used to count cell number.

2.4 Cellular cytotoxicity assessment

Cell viability was determined using a cell counting kit (CCK-8, Dojindo Molecular Technologies, Inc). Briefly, cells were harvested from culture dishes and seeded in 96-well plates with 5×10^3 cells/well at 37°C in a humidified 5% CO_2 environment. The cells were exposed to medium containing 0, 10, 50, 100, 250 and 500 µg/mL of QRH*-KSP*-E3-IRDye800 for 48 hours (n=3). CCK8 (10 µL) was added to each well, and incubated for 2 hours. Cell absorbance was

measured with a microplate spectrophotometer at λ_{abs}=450 nm. The experiments were performed in triplicate. The values were normalized by the controls and expressed as percent viability.

2.5 In vitro heterodimer validation

~10^4 human cancer cells, including SKBr3, OE21, OE19,and QhTERT, were seeded on 1 mm thick cover glass and grown to ~80% confluence in 12 well plates. The cells were washed with PBS and blocked with 2% BSA, followed by incubating with 1 μM QRH*-KSP*-E3-IRDye800 for 10 min at RT. The cells were washed with PBS 3X, fixed in ice cold PFA for 10 min, and washed with PBS. The cells were then embedded on microscope slides with 10 μL of Prolong Gold reagent containing DAPI (Invitrogen).

2.6 Xenograft tumor

All experimental procedures were performed in compliance with relevant guidelines and regulations of the University of Michigan, and all animal studies were conducted with approval by the University Committee on the Use and Care of Animals (UCUCA). Animals were housed per guidelines of the Unit for Laboratory Animal Medicine (ULAM). Tumors were established in female nude athymic mice (002019 Foxn1 <nu>, Jackson Laboratory) at 4-6 weeks of age with weight between 20-25 grams. ~5×10^7 OE33 cells were suspended in 100 μL of sterile PBS and injected subcutaneously into the hind limb flank. In vivo imaging experiments were performed when the tumors reached 0.5 cm in dimension at ~2-3 weeks.

2.7 In vivo heterodimer validation

OE33 tumor bearing mice were injected intravenously with QRH*-KSP*-E3-IRDye800 (300 μM, 200 μL). The spatial extent and margins of tumors were identified using a NIR whole body fluorescence imaging system (Pearl®, LI-COR Biosciences). The images were acquired

using λ_{ex} = 800 nm with 85 μm resolution and 16.8×12 cm^2 FOV. Custom software (Image Studio, Li-Cor Biosciences) was used for analysis. Regions of interest (ROI) with area equal to that of the tumor and adjacent in location was measured for background. The animals were sacrificed 2 hours after intravenous injection of the heterodimer and control peptide, and major organs, including heart, spleen, lung, liver, brain, stomach, kidney, intestine and muscle, were resected to measure biodistribution. For photoacoustic imaging, three-dimensional (3D) images were reconstructed off-line using data acquired from all 128 transducers at each view and a back-projection algorithm. The photoacoustic signal intensity was measured from the 2D MIP (Maximum Intensity Projection) images, and the pre-injection image was used for background.

2.8 Ex vivo heterodimer validation

Formalin-fixed, paraffin-embedded (FFPE) specimens of human OE33 xenograft tumor were prepared by using standard methods followed by deparaffinization, and rehydration. Antigen unmasking was performed using two-step acidic and protease-activation. The sections were blocked with protein serum for 10 min at RT followed by washing with PBS, and then incubated with 0.5 μM of heterodimer for 10 min in dark conditions at RT. The sections were then washed 3X for 5 min each with PBS and further incubated sequentially with 1:200 dilution of EGFR monoclonal antibody (H11, #MA5-13070, Invitrogen) and 1:500 goat anti-mouse IgG (H+L) AF-568-labeled secondary antibody (#A-11004, Thermo Fisher Scientific), 1:500 dilution of HER2 primary antibody (#29D8, rabbit monoclonal antibody #2165S, Cell Signaling Inc) and 1:1000 goat anti-rabbit IgG (H+L) AF-488-labeled secondary antibody (#A11008, Life Technologies Corp). Each antibody was incubated for 1 hour at RT. Sections were washed with TBST 3X and mounted with Prolong Gold reagent containing DAPI (Invitrogen) using #1 cover glass (1.5 μm thickness). Confocal fluorescence microscopy (Leica Inverted SP5) was performed using DAPI,

AF488, AF568 and Cy5.5 filter sets. Image quantification was performed by randomly placing 3 boxes with dimensions of 20×20 μm^2 completely within tumor and normal areas. Regions of saturated intensities were avoided. Mean fluorescence intensities were measured using custom Matlab software.

Tumor-bearing mice were euthanized, and the cancers were excised followed by fixation in 10% buffered formalin and paraffin-embedded (FFPE). Sections were cut with 10 μm thickness and deparaffinized. Rehydration was performed using a standard protocol, and antigen retrieval was performed by boiling the slides in 10 mM sodium citrate buffer with 0.05% Tween at pH 6.0 followed by a sub-boiling temperature for 15 min. The slides were washed 3X with dH_2O after cool down. Endogenous peroxidase activity was quenched by incubating the sections with 0.3% hydrogen peroxide in methanol for 30 min. The slides were washed in dH_2O and blocked with protein blocking buffer for 30 min at RT. After washing 1X with PBS, the slides were incubated with either anti-EGFR (1:500 dilution, H11, #MA5-13070, Invitrogen) or anti-HER2 (1:500 dilution, #29D8; rabbit monoclonal antibody #2165, Cell Signaling Inc) primary antibody overnight at 4°C in a humidified chamber and then washed 3X for 10 min each with PBS. HRP-labeled secondary antibodies, including goat anti-mouse IgG for EGFR and goat anti-rabbit IgG for HER2 at 1:200 dilution, was added to each section and incubated for 30 min at RT followed by washing 3X for 10 min each with PBST. The sections were incubated with premixed Elite Vectastain ABC reagent for 30 min at RT and then washed 3X for 10 min each with PBS. The sections were developed with 3,3'-diaminobenzidine substrate (DAB) and incubated for 3 min. The slides were washed 2X for 3 min each with PBST. Hematoxylin was added as a counterstain for 20 sec, and the sections were dehydrated in ethyl alcohol. Sections were mounted on slides with coverslip in xylene. Serial sections were processed for histology (H&E).

2.9 Statistical analysis

The intensities of fluorescence and photoacoustic were measured by region of interest (ROI) analysis using Pearl® system, LI-COR Biosciences and OsiriX respectively. Results were expressed as the mean ± standard deviation. Statistical comparisons between two groups were determined by t-test. Prism (v6.02, GraphPad) and OriginPro Softwares 8.5.1 were used to perform statistical analysis and plot data.