Supporting Information for *M13 phage-functionalized single-walled carbon nanotubes as nanoprobes for second near-infrared window fluorescence imaging of targeted tumors*

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Materials and Methods

M13 phage-SWNTs complexation

To prepare the starting SWNTs solution, as-produced and non-acid treated HiPCO single-walled carbon nanotubes, purchased from Unidym, was diluted in a 2wt% sodium cholate (SC) aqueous solution. The diluted solution was homogenized for 1 h, cup-horn sonicated for 10 min at 90% amplitude and then centrifugated at 30,000 rpm for 4 h to get individually dispersed SWNT. SWNT concentration was calculated using the extinction coefficient of HiPCO SWNT at 632 nm, $\varepsilon_{632\ nm}$=0.036 L/mg•cm (or $A_{632@1cm}$ × 27.8=[SWNTs] in μg/mL). For the complexation, a phage-to-SWNT ratio of 1:1 was used. The complexation was done according to previously reported method[S1]. Briefly, calculated amount of SWNT-binding phage solution was mixed with the calculated volume of SWNTs dispersed by 2 wt% SC in water. The mixed solution was dialyzed against water (10 mM NaCl, pH =5.3, which is pI of SWNT-binding phage) for two days with frequent solution changes, and the pH of the dialyzing solution was increased to 10 after two days of dialysis. A dialysis membrane with MWCO of 12,000-14,000 (SpectraLabs) was used for the dialysis. After the complexation, concentrated PBS (10 X PBS) was added to the complexes, and the complex solution was vortexed and centrifuged at 6,000 rpm for 5 min.

Absorption spectroscopy and NIR photoluminescence excitation (PLE) mapping

Absorption measurements were taken with a Shimadzu UV-3101 PC UV-VIS-NIR Scanning Spectrophotometer. PL from SWNT was measured with a home-built near-infrared (NIR) PL microscope. An inverted microscope was coupled to OMA V 1D InGaAs array detector (Princeton Instruments) through Acton SP2500 spectrometer (Princeton Instruments). For three-dimensional profile, a Xe lamp coupled to a monochromator was used as excitation source.

Genetic engineering of SPARC binding peptide (SBP) onto p3 of SWNT-binding M13 phage

SPARC binding peptide (designated as SBP), SPPTGINGGG[S2], was used for specific binding to SPARC. Oligonucleotides encoding SBP, 5'(Phos)-GTA CCT TTC TAT TCT CAC TCT TCA CCA CCG ACT

GGA ATT AAC GGA GGC GGG TC -3' and 5'(Phos)-GGC CGA CCC GCC TCC GTT AAT TCC AGT CGG TGG TGA AGA GTG AGA ATA GAA AG-3' (IDT) were annealed to form a DNA duplex. The M13-based cloning vector was isolated from the SWNT-binding phage (designated as DSPH) using standard miniprep kit (QIAGEN). DNA was digested with Eag I and Acc65 I restriction enzymes, dephosphorylated and agarose-gel purified. Purified vector and DNA duplex were ligated using T4 DNA ligase at 16°C overnight and transformed in electrocompetent XL-1 Blue cells (Stratagene). Transformed cells were incubated for 1 h and plated in top agar and incubated at 37°C overnight. Blue plaques were amplified, and isolated DNA was purified and sequenced to confirm the insertion of oligonucleotides to express SBP on p3.

Genetic engineering for biotin-accepting peptide (BAP) onto p3 of SWNT-binding M13 phage

The genetic engineering of BAP^{S3} is identical to the cloning of SBP described above, except that oligonucleotides sequences, 5'(Phos) GTA CCT TTC TAT TCT CAC TCT GGC CTG AAC GAC ATC TTC GAG GCT CAG AAA ATC GAA TGG CAC GAG TC 3' and 5'(Phos) GGC CGA CTC GTG CCA TTC GAT TTT CTG AGC CTC GAA GAT GTC GTT CAG GCC AGA GTG AGA ATA GAA AG 3', were used to make a DNA duplex encoding BAP.

Cell lines and culture

DU145 human prostate carcinoma cell line was provided courtesy of Dr. Kimberly Kelly (University of Virginia). LNCaP human prostate carcinoma cell line was purchased from ATCC. DU145 was grown in Dulbecco's Minimum Essential Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin (Invitrogen) at 37°C in 5% CO_2. LNCaP were grown in phenol red-free RPMI medium (Hyclone), supplemented with 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate (Invitrogen), and 1% HEPES buffer (Invitrogen) 37°C in 5% CO_2.

Flow cytometry

To determine SPARC expression, DU145 and LNCaP cells were harvested. 1,000,000 cells/sample were incubated with complete media and spun at 1,200 rpm for 5 min. After centrifugation, samples were

washed two times with PBS and fixed with cold 4% paraformaldehyde for 10 min at room temperature. After two washes, cells were washed with 0.2% saponin in PBS (SAP) for 10 min. Cells were centrifuged at 1,200 rpm for 5 min. Cells were then incubated with 1: 20 mouse isotype (abcam) or anti-human SPARC-phycoerthrin (PE) (R&D Systems) in SAP buffer. After washes, samples were run on FACScan (Becton Dickinson) and gated for 10,000 events. Samples were run in triplicate. For PSMA expression, DU145 and LNCaP were harvested and washed once with PBS. After centrifugation, samples were incubated with 1:100 mouse isotype-PE (abcam) or mouse anti-PSMA-PE (abcam). After 30 min, samples were washed twice with PBS and run on FACScan. For each experiment, 10,000 events were gated. Samples were run in triplicate. All analysis was done using FlowJo software.

Mouse handling

All animal handling and procedures were done in accordance with Institutional Animal Care and Use Committee protocols. For tumor studies, human xenograft prostate tumors were induced in six-to-eight week old male nude *nu/nu* mice (Charles River Laboratories). Mice were subcutaneously injected in the right flank with 3,000,000-4,000,000 LNCaP cells suspended with equal volume of Matrigel (BD Biosciences). Tumors were grown until they reached 3-7 mm in diameter. Mice were dosed with M13-SWNT probes by retro-orbital injection. For *ex vivo* analysis, mice were sacrificed at 24 h p.i. and organs were collected, measured and weighed.

Blood circulation study

For blood circulation, a few μl of blood was collected using a quartz capillary tube at each time point, and the NIR PL intensity of the blood samples was measured using the home-built imager (Supplementary Fig. S1), described above. For quantitative analysis of the blood samples, a collimated laser was used. The actual fluence of the collimated laser on the sample was ~ 170 mW/cm^2 and the acquisition time was 0.5 s. To calculate %ID/g of SWNTs in blood, an equation, %ID/g={$[SWNTs]_{blood}$ x V_{blood} x 100} / {$[SWNTs]_{injected}$ x $V_{injected}$ x W_{blood}} was used. V_{blood} and W_{blood} were measured from each sample[S4]. The SWNTs concentration was calculated using a calibration curve (Supplementary Fig. S2). For circulation study, three mice were used.

Near-infrared fluorescence whole-animal imaging

An *in vivo* imager has been built for this study (Supplementary Fig. S1). As a detector, a liquid nitrogen-cooled OMA V 2D InGaAs array detector (detection range: 800 – 1,700 nm) with a 256 × 320 pixel array (Princeton Instruments) was used. In front of the detector, NIR camera lens (SWIR-25, Navitar) was attached. To minimize autofluorescence from tissues and maximize the detection of fluorescence from SWNTs, two stacked long-pass filters with cut-off wavelength of 1,100 nm and OD >4 (EdmundOptics) were used. For the excitation, an optical fiber coupled 808 nm diode laser (MDL-F-808, OptoEngines) was used and a laser line filter with center wavelength of 808 nm (EdmundOptics) was attached in front of the laser to remove any unwanted excitation light. To minimize the exposure of the laser onto the mouse, a computer-controlled shutter was set-up. The actual fluence on the mouse for *in vivo* imaging was ~120 mW/cm^2. The acquisition time for *in vivo* imaging was 0.1 s ~ 1 s. For the contrast images, the same detector was used but a white light was illuminated instead of an 808 nm laser.

Near-infrared fluorescence imaging of M13-SWNTs in phantoms

In the phantom study, to get depth information of the probe, a stacked 3D image was constructed. Phantoms were scanned along the depth direction with a scanning velocity of 0.5 mm/s, controlled by two-axis traveling stage controller (10 mm maximum travel length in each direction, Thorlabs), with a collimated laser of ~5 mm in diameter (collimator, F230SMA-B, Thorlabs), and fluorescence images of the phantom were collected every 0.5 s and were used to construct a 3D stacked image after background subtraction (Fiji, freeware).

***In vitro* binding assay**

To compare various binding ligands of M13-SWNTs, a binding assay was done using the imager. In a poly-lysine coated, black 96-well plate, 30,000 cells (either LNCaP or DU145) were plated in each well and 100 μL of complex solution with a concentration of 10^{12}/mL (SWNTs concentration: ~ 1μg/mL) was added to each well, incubated at 37°C for 4 h. After incubation, wells were washed three times with PBS and PL was measured. PL intensity was averaged over three wells. The acquisition time was 1 s for all samples.

Near-infrared fluorescence microscopy

For NIR fluorscence imaging of sectioned tumor tissues, samples were excited by 658 nm laser and imaged and monitored using inverted microscope with liquid nitrogen-cooled OMA V 2D InGaAs detector and an AxioCam MRm charge-coupled device (CCD) camera.

Immunohistochemistry

Tissues and tumors were harvested, embedded in OCT resin and snap frozen in dry ice. Samples were cut into 5 μm sections. Immunostaining was done using ThermoScientific Autostainer 360. For immunostaining, sections were blocked with 3% H_2O_2 and blocked for endogenous mouse IgGs prior to incubation with 1:150 mouse anti-PSMA (Lifespan Biosciences) in PBS. Sections were then incubated with secondary horseradish peroxidase conjugate (ThermoScientific) and DAB chromogenic substrate (Ultravision). Samples were imaged using Olympus IX51 inverted microscope.

Supplementary figures

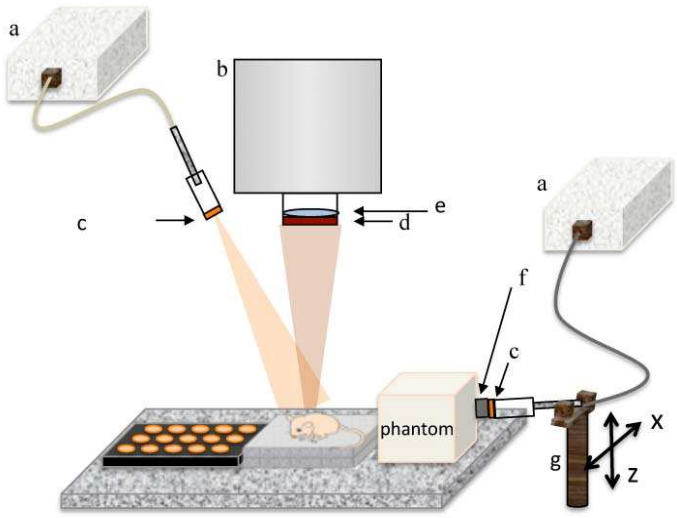

Fig. S1. A schematic of our second near-infrared window fluorescence imager. The versatile imaging capability allows for *in vivo* imaging as well as *in vitro* screening and 3D stacked imaging of optical phantoms. a: 808 nm diode laser, b: liquid-nitrogen cooled two-dimensional InGaAs detector, c: 808 nm laser line filter, d: 1,100 nm long-pass filter, e: lens for NIR light, f: collimator, and g: stage controller for x- and z- directional travel of the laser.

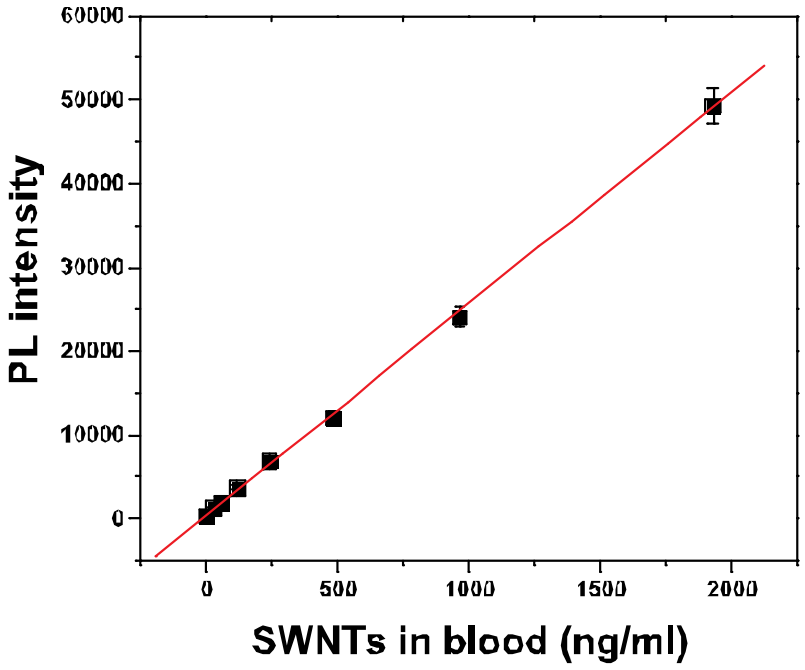

Fig. S2. Calibration curve for the SWNT concentration in blood used for circulation study. The acquisition time was 0.5 s and the excitation fluence was ~ 120 mW/cm^2. The fitted line is PL intensity=392.8 + 25.266 × [SWNTs] in ng/mL.

References

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