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Supplementary Information for

High Precision Tumor Resection Down to Few-Cell Level Guided by NIR-IIb Molecular Fluorescence Imaging

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Movies S1 to S6

Supplementary Information Text

Synthesis of ErNPs. Rare-earth(III) acetate hydrate (RE: Yb, Er, Ce), zinc acetate, sodium trifluoroacetate, oleic acid, ODE, sodium hydroxide, ammonium fluoride, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), Poly(acrylic acid) (PAA, average molecular weight: 1,800), Poly(maleic anhydride-alt-1-octadecene) (PMH, average molecular weight: 30k–50k), 4-morpholineethanesulfonic acid (MES), 4-(dimethylamino)pyridine (DMAP), cyclohexane, chloroform, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-base) were bought from Sigma-Aldrich and used without further purification. Yttrium (III) oxides and trifluoroacetic acid (99%) were obtained from Alfa Asear. mPEG-NH_2 (average molecular weight: 5k) was bought from Laysan-Bio. 8Arm-PEG-NH_2 (average molecular weight: 40k) was purchased from Advanced Biochemicals. $Y(CF_3COO)_3$ was prepared according to the method described in the literature[1].
Synthesis of α-$NaYbF_4$:Ce,Er,Zn (core) and subsequently the $NaYF_4$ shell to afford the final α-$NaYbF_4$:Ce,Er,Zn@$NaYF_4$ (core-shell ErNPs) nanoparticles followed the steps described in Ref. 2. The synthesis led to ErNPs coated with oleic acid dispersed in 3 ml cyclohexane with a mass concentration of ~ 80 mg/ml.

Surface modification of ErNPs with P^3 coating. To transfer the ErNPs to aqueous phase, we first coated the particles with PMH (Poly(maleic anhydride-alt-1-octadecene), 30–50 kDa, 419117-250G, Sigma-Aldrich) in chloroform, dried and then added to water with DMAP (4-(dimethylamino)pyridine, 107700-25G, Sigma-Aldrich) under sonication to afford COOH coated ErNPs. The particles were then conjugated to 8Arm-PEG-NH_2 (8AP0804, Advanced BioChemicals) that cross-linked with the PMH on the particles by EDC chemistry, then cross-linked to a PAA (Poly(acrylic acid), 323667-5G, Sigma-Aldrich) layer through EDC chemistry, and finally cross-linked with another layer of 8Arm-PEG-NH_2 to afford the P3 coated ErNPs in aqueous solution. Detailed steps are described in Ref. 2 with a modification that EDC reactions were done in pH = 11 MES solutions instead of pH = 8.5. The final ErNPs (~16 mg) with P^3 coating were dispersed in 1.6 ml 1x PBS solution and kept at 4 °C for further application.

Zeta potentials of free P^3-ErNPs and ErNPs-TRC105 were measured by Nanobrook Omni particle size and zeta potential analyzer (Brookhaven Instrument).

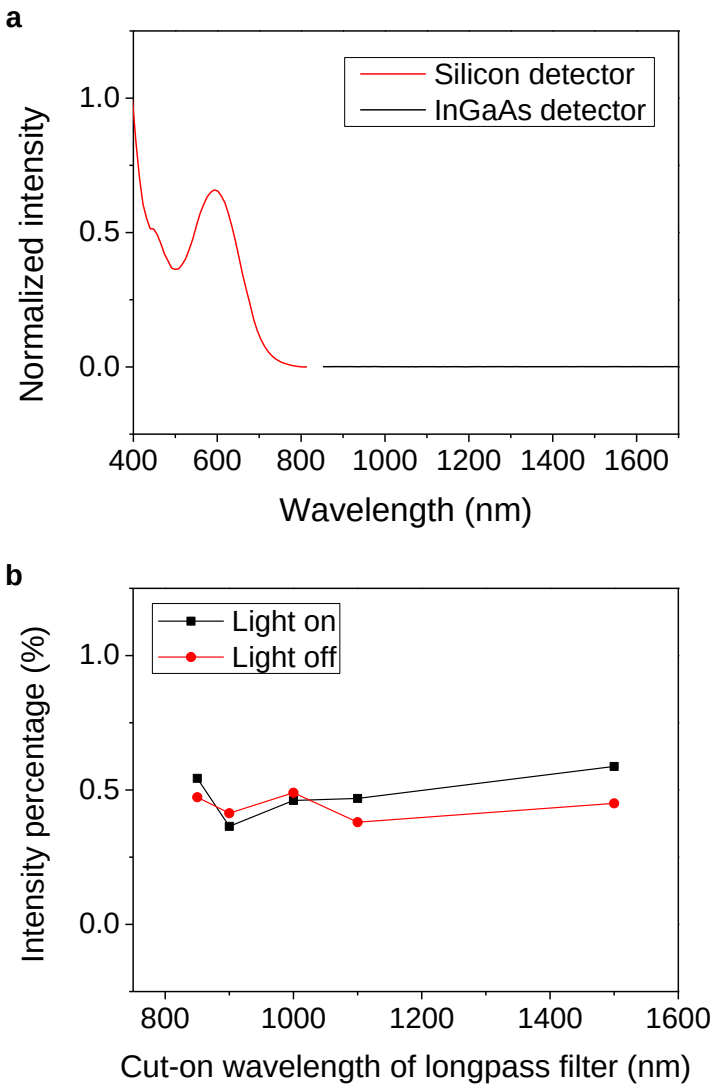

Fig. S1. Influence of the room light on the NIR-I and NIR-II imaging. (**a**) Spectrum of the RGB LED for room lighting measured by a silicon detector in visible window and an InGaAs detector in NIR-I and NIR-II windows. (**b**) The y-axis shows the percentage of the averaged background with room light “on” or “off” to the full well depth (65535). The x-axis shows the cut-on wavelength of longpass filters used in NIR-I or NIR-II imaging.

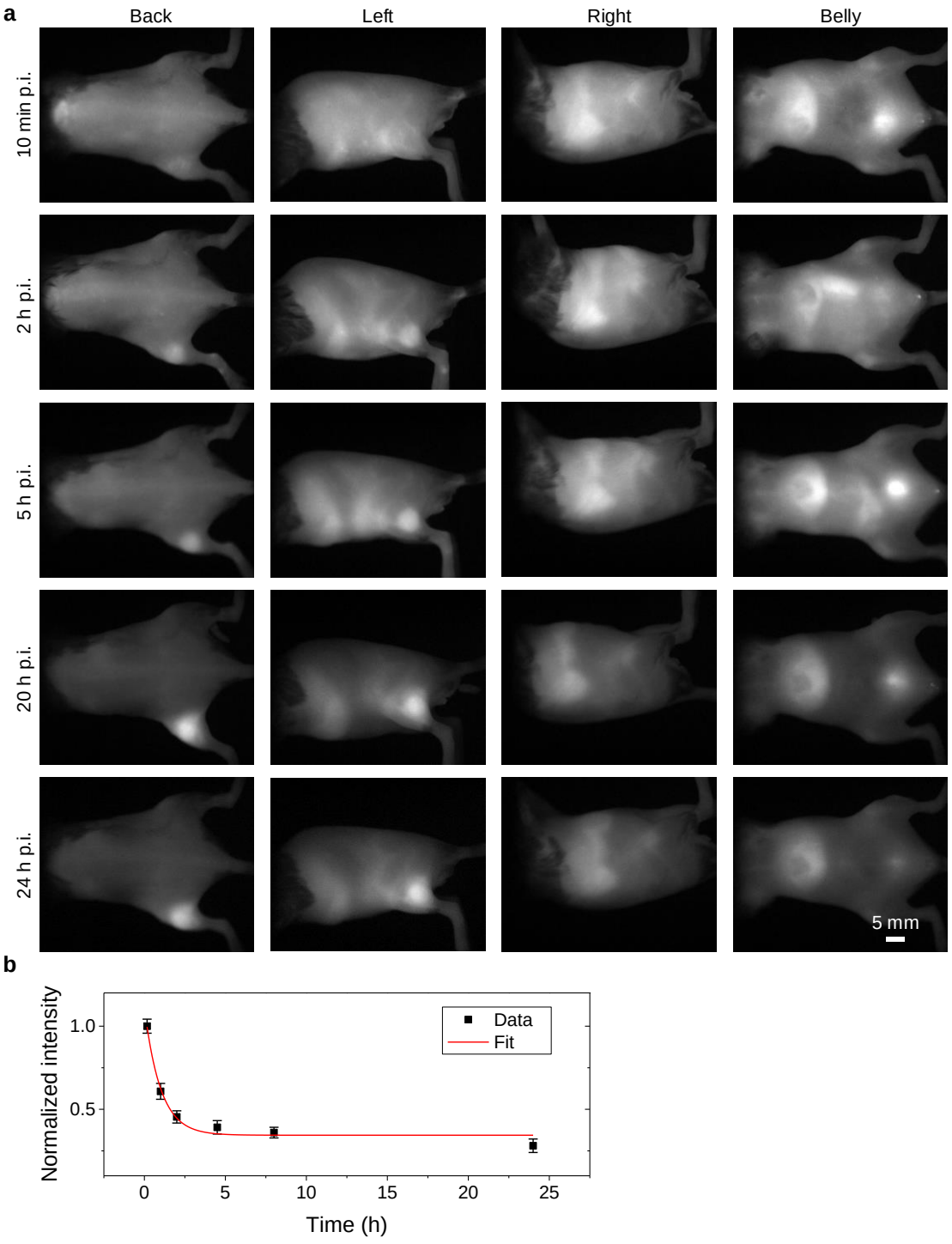

Fig. S2. Time-course NIR-I (900-1000 nm) fluorescence imaging of a Balb/c mouse bearing a 4T1 tumor injected with IRDye800-TRC105. (**a**) Additional images corresponding to the NIR-I molecular imaging in Fig. 2a and showing the in vivo bio-distribution of IRDye800-TRC105 in the whole body. The body signal was still strong 24 hours post injection. (**b**) NIR-I fluorescence signal in femoral artery of the mice at different time points post injection. The calculated blood circulation half-time was ~ 0.6 h.

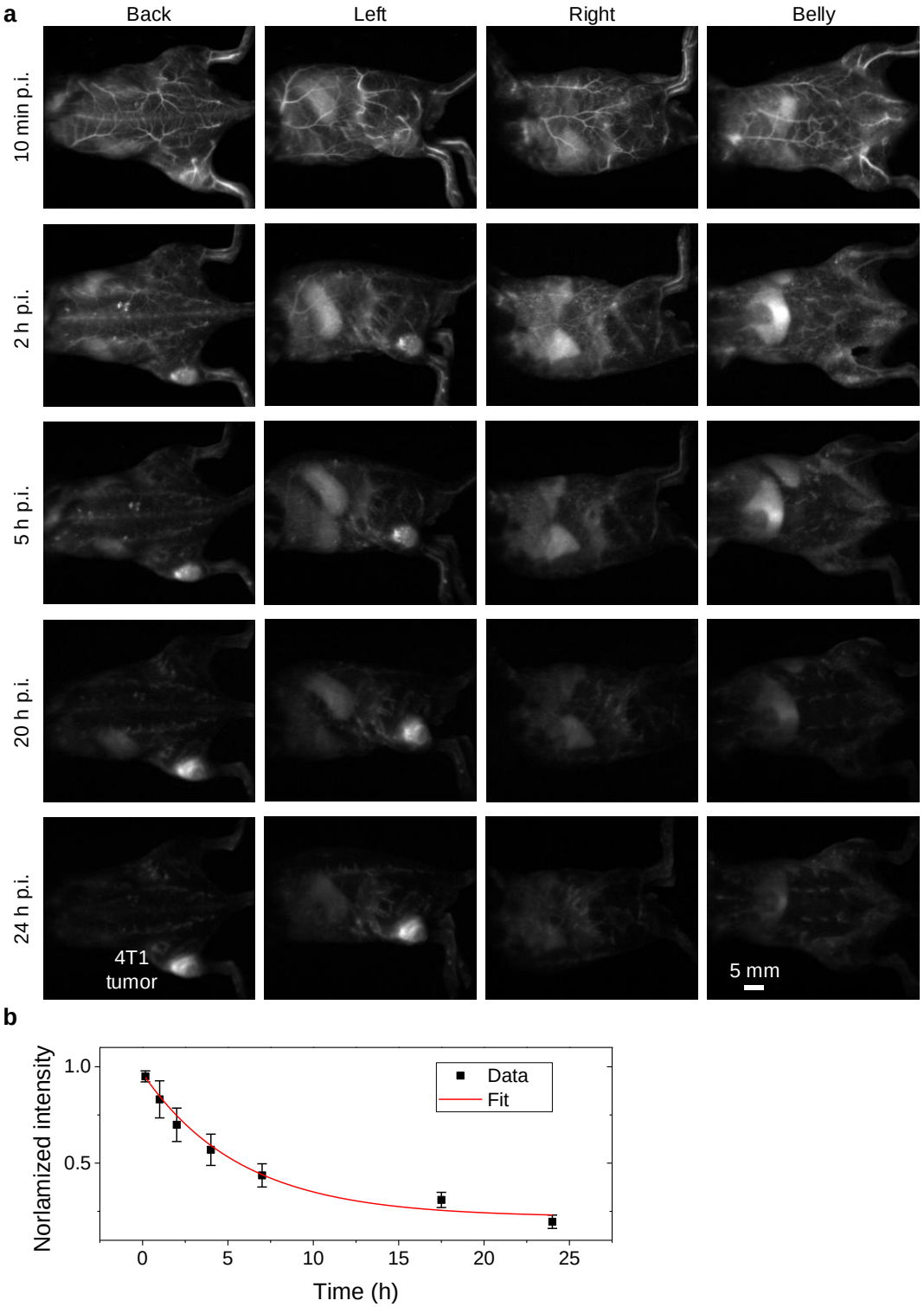

Fig. S3. Time-course NIR-IIb fluorescence imaging of a Balb/c mouse bearing a 4T1 tumor injected with ErNPs-TRC105. (**a**) Additional images corresponding to the NIR-IIb molecular imaging in Fig. 2a and showing the in vivo bio-distribution of ErNPs-TRC105 in the whole body. The body signal was almost undetectable 24 hours post injection. (**b**) NIR-IIb fluorescence signal in femoral artery of the mice at different time points post injection. The blood circulation half-time was ~ 4 h.

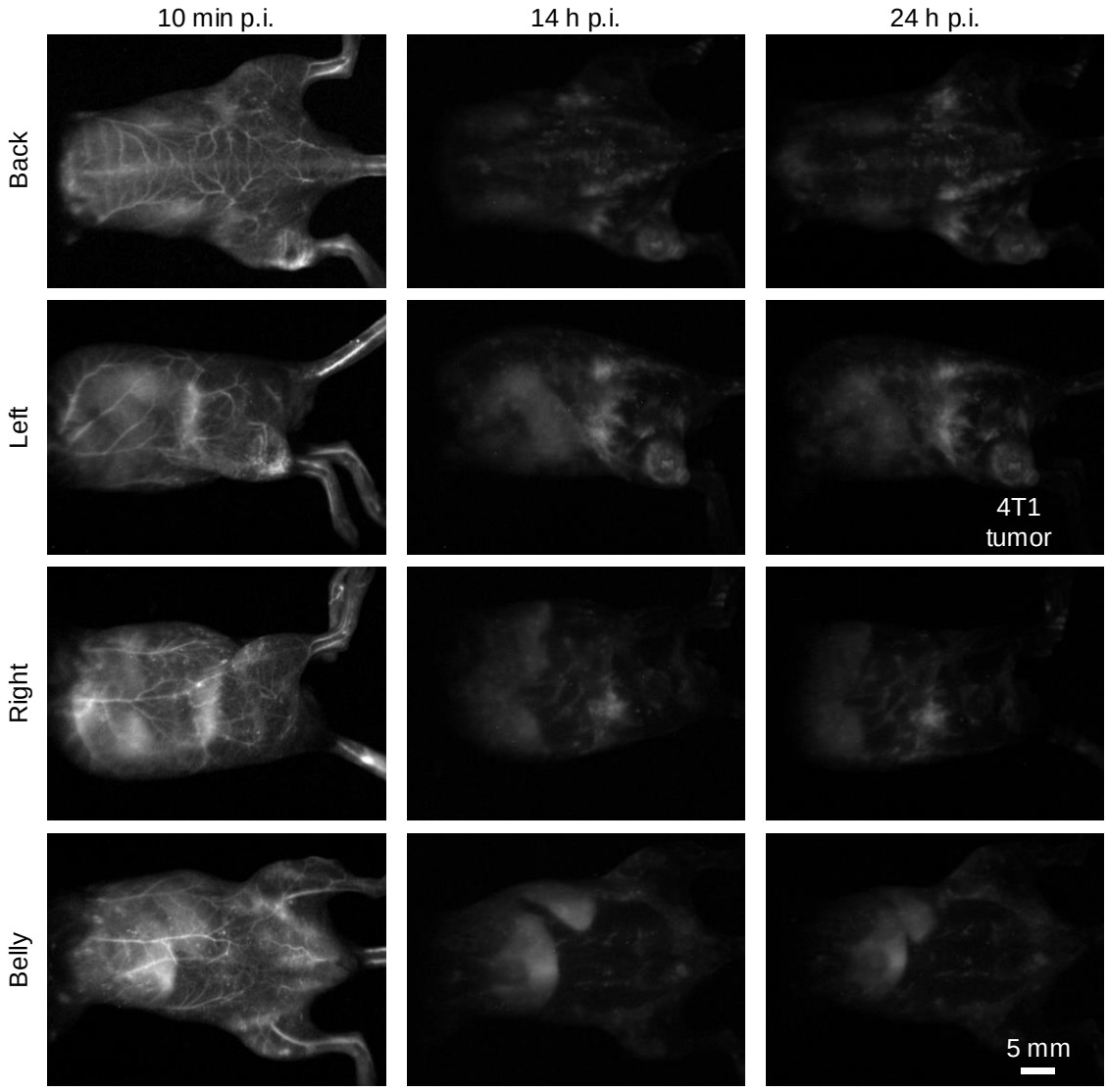

Fig. S4. Wide-field images of a Balb/c mouse recorded 10 min, 14 h and 24 h after intravenous injection of free ErNPs. The fluorescence was collected in the 1500-1700 nm under a 975 nm laser excitation. 24 hours post injection, the T/NT ratio was ~ 10.

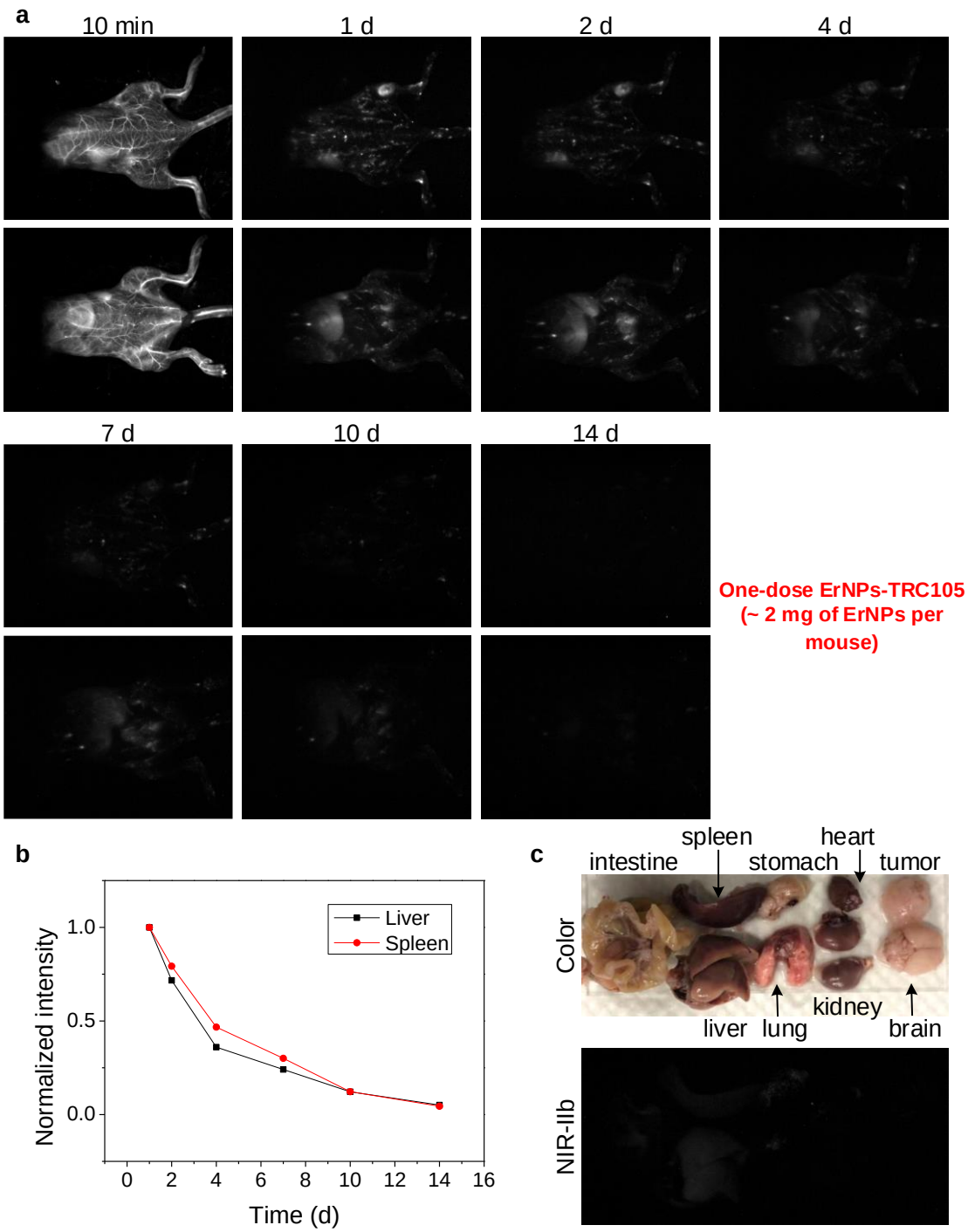

Fig. S5. (a) NIR-IIb fluorescence imaging of a Balb/c mouse over 2 weeks post intravenous injection of one-dose ErNPs-TRC105 (~ 2 mg of ErNPs per mouse). One dose of ErNPs-TRC105 conjugates was injected intravenously through the tail vein when the tumor size reached ~ 4 mm. (b) Fluorescence signal of liver and spleen over the course of 2 weeks. (c) Wide-field color (upper) and NIR-IIb (lower) fluorescence images of major organs 14 days after the injection of ErNPs-TRC105.

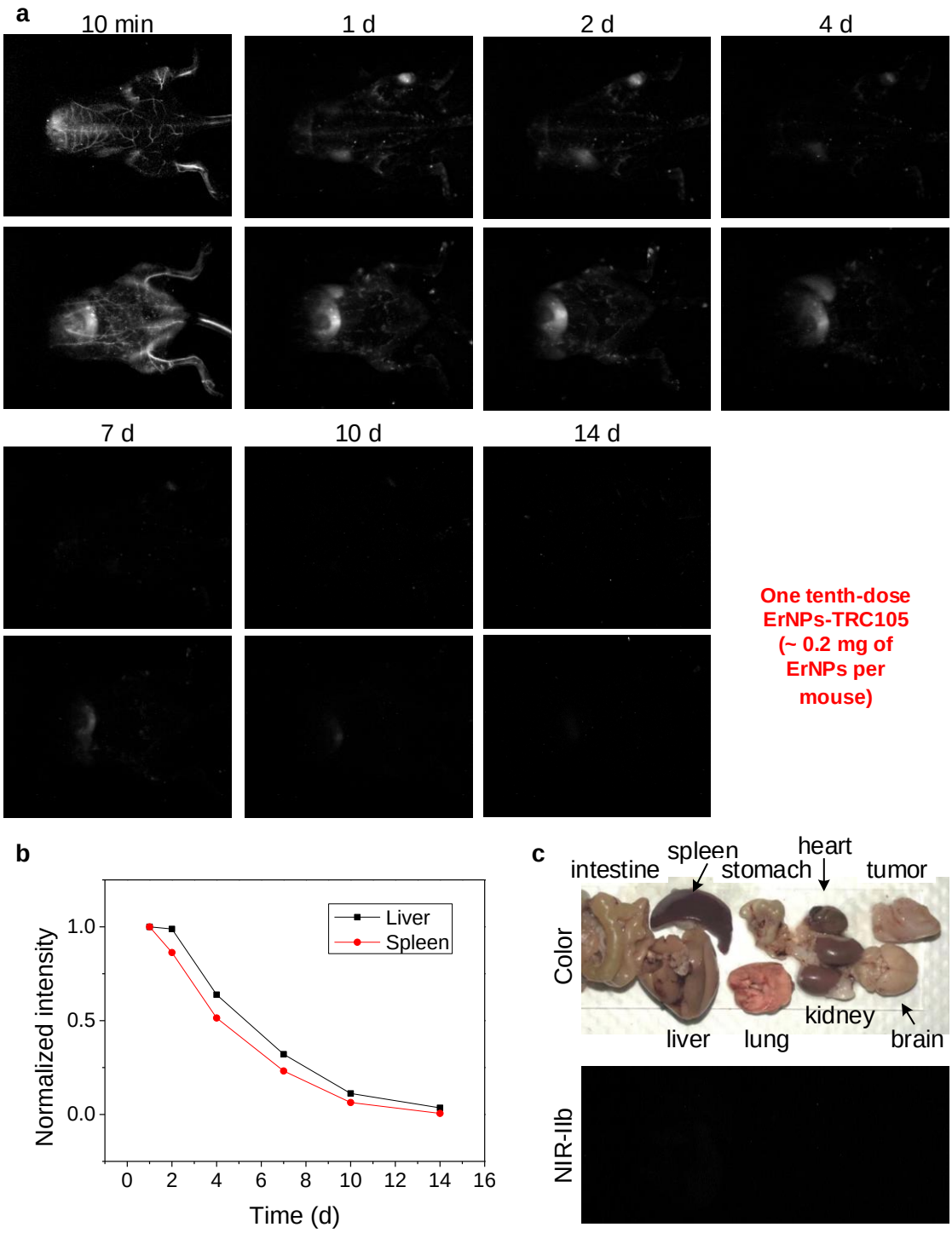

Fig. S6. (**a**) NIR-IIb fluorescence imaging of a Balb/c mouse over 2 weeks post intravenous injection of one tenth-dose ErNPs-TRC105 (~ 0.2 mg of ErNPs per mouse). One tenth dose of ErNPs-TRC105 conjugates was injected intravenously through the tail vein when the tumor size reached ~ 4 mm. (**b**) Fluorescence signal of liver and spleen over the course of 2 weeks. (**c**) Wide-field color (upper) and NIR-IIb (lower) fluorescence images of major organs 14 days after the injection of ErNPs-TRC105.

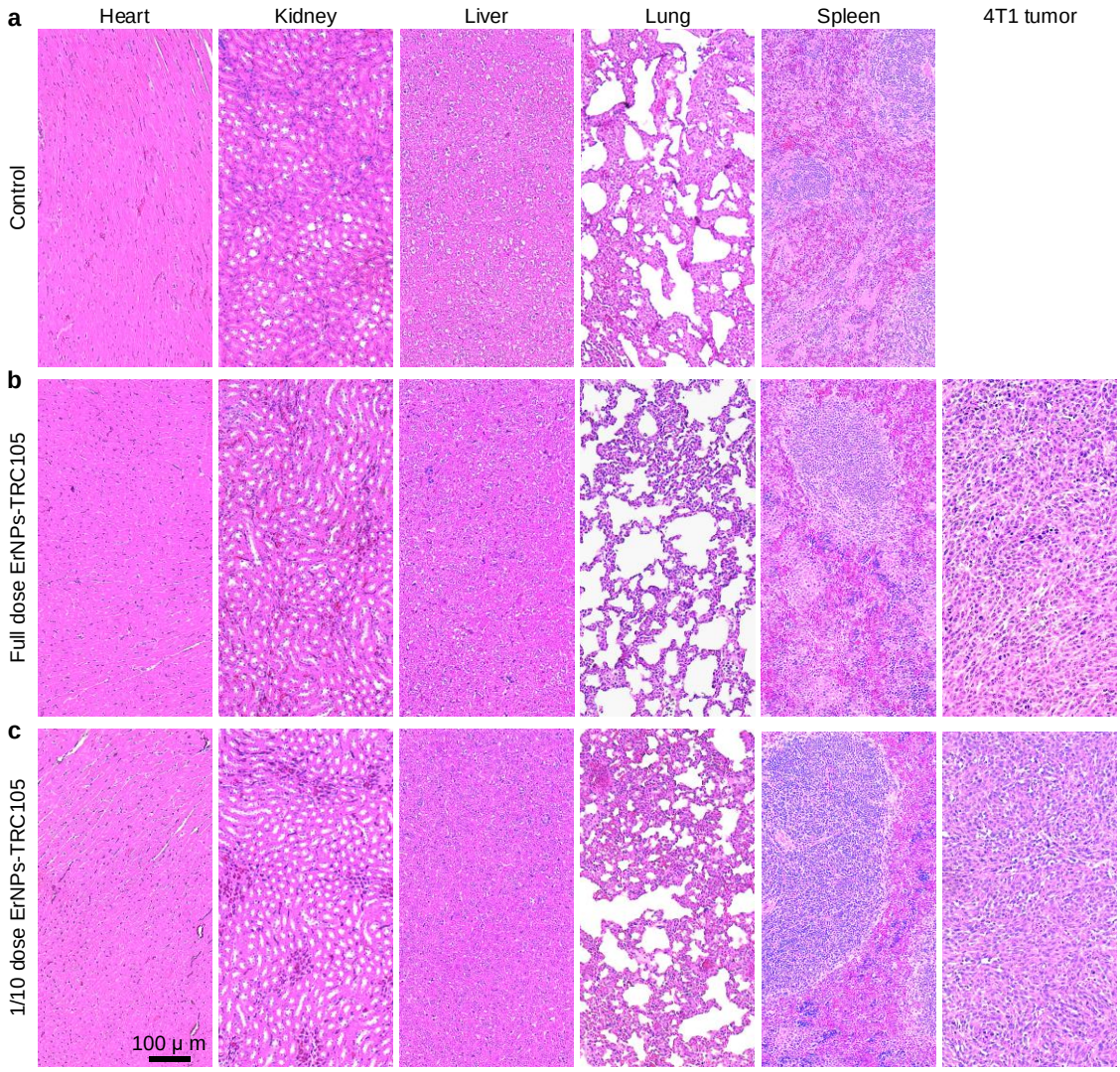

Fig. S7. Histological studies of hematoxylin and eosin (H&E). H&E stained histological sections of the main organs from (**a**) a healthy mouse and a mouse injected with (**b**) one-dose ErNPs-TRC105 or (**c**) one tenth-dose ErNPs-TRC105 (~ 0.2 mg of ErNPs per mouse) 2 weeks post injection. No detectable injury or difference was observed thus illustrating no apparent toxic effects.

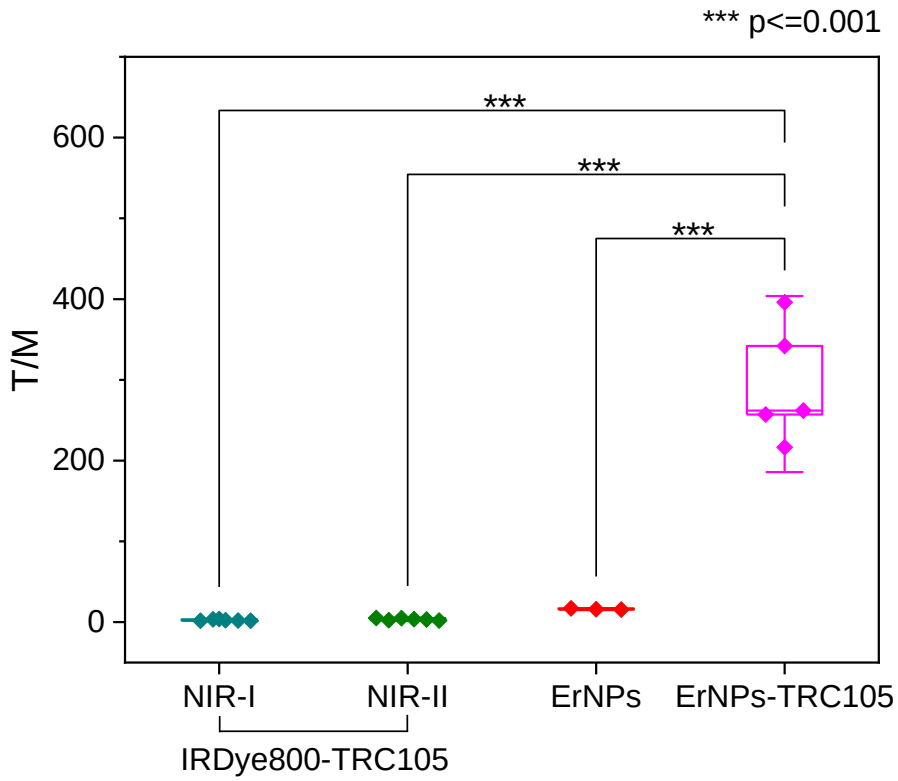

Fig. S8. Tumor-to-muscle ratios of mice injected with IRDye800-TRC105 (n = 6), free ErNPs (n = 3) and ErNPs-TRC105 (n = 5) 24 hours post injection as shown in Fig. 3c and Fig. 4c. Data are presented as box plots (center line, median; interquartile range, 25th and 75th percentile; whiskers, 1.5 × s.d.; points, outliers; ***, $p \leq 0.001$, Tukey's test).

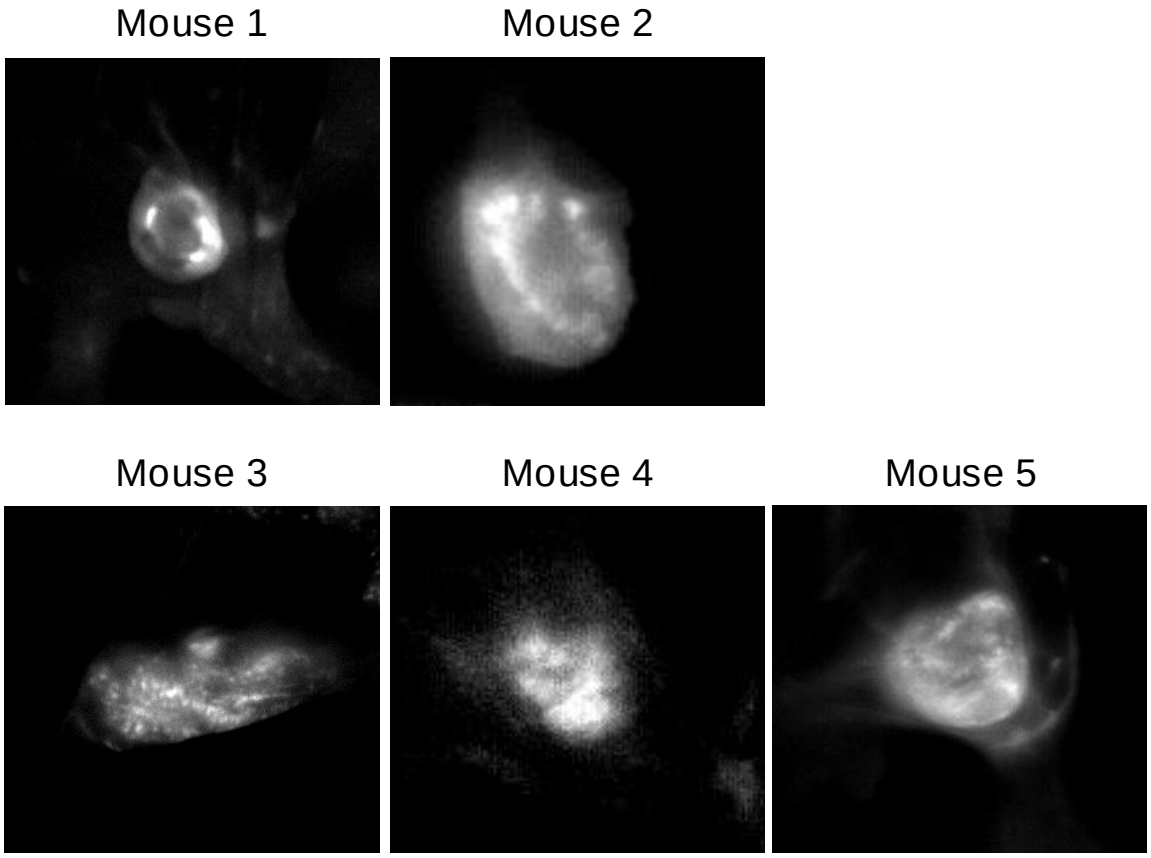

Fig. S9. 4T1 tumor imaging in the NIR-IIb window 24 h post intravenous injection of ErNPs-TRC105. Images were recorded during surgery after skin removal. Fluorescence was excited by a 940-nm LED. NIR-IIb emission was collected in the 1500-1700 nm window.

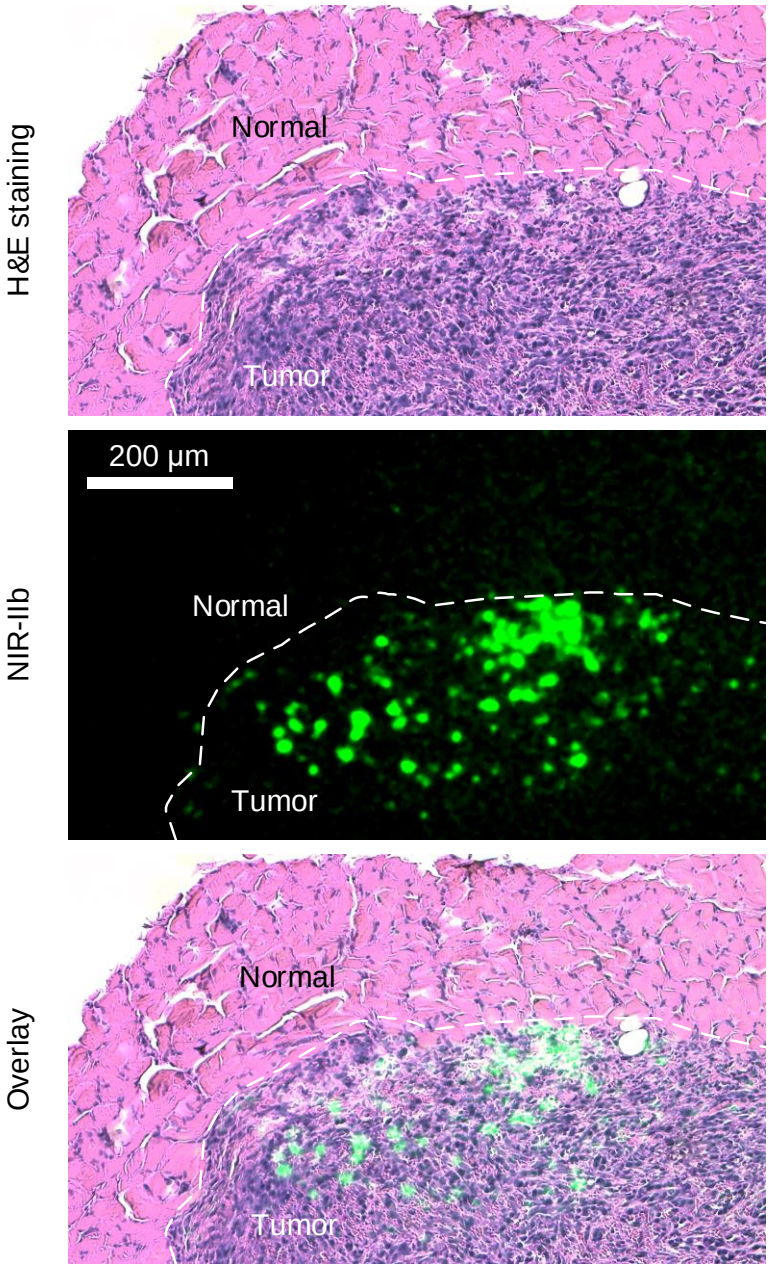

Fig. S10. Two channel (upper) H&E and (middle) NIR-IIb imaging of tumor slices resected 24 h post intravenous injection of ErNPs-TRC105. The injection was performed through the tail vein when the tumor size reached ~ 4 mm (typically 4 d after inoculation). The tumor was taken out 24 h post intravenous injection of ErNPs-TRC105, followed by tumor slicing and H&E staining by standard procedure. We first imaged the slices in NIR-IIb window by using a home-made NIR-II microscope with a 5X objective. ErNPs-TRC105 was excited by a 975-nm laser and the emission was collected in the 1500-1700-nm window. Then we removed the filters and imaged the same position using the same NIR-II grey camera with a white light illumination. This white-light image was used as a guide to locating the same region during H&E imaging using a commercial Leica microscope (DM2700 M). The H&E image was scaled and shifted to overlap with the grey image recorded by the NIR-II camera under white light illumination. NIR-IIb and the resulting H&E images were then overlaid in ImageJ. Note that the tumor slices were ultra-thin (~ 5 μm) and did not contain large numbers of ErNPs-TRC105 in each slice.

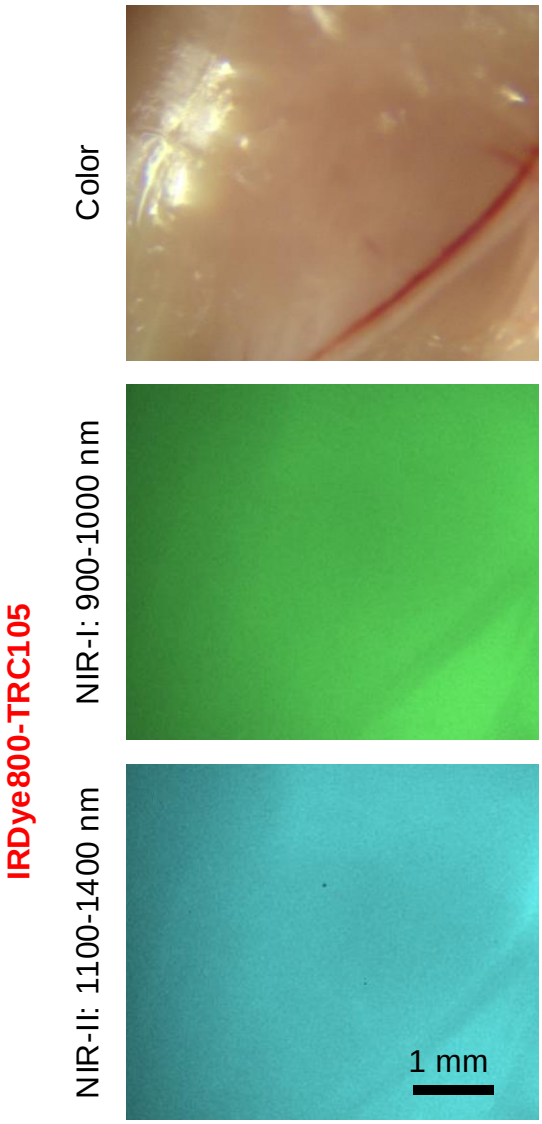

Fig. S11. High-resolution NIR-I and NIR-II imaging of the surgery area after tumor removal. When the 4T1 tumor size reached ~4-8 mm, IRDye800-TRC105 was injected intravenously. Surgery was performed 24 h post injection of IRDye800-TRC105. The fluorescence was collected in 900-1000 nm for NIR-I imaging or in 1100-1400 nm for NIR-II imaging excited by an 808-nm laser. A 5X objective (NA = 0.12, FOV: 5.8 x 4.7 mm^2) was used.

Movie S1 (separate file). Molecular image-guided surgery of cutting the skin covering 4T1 tumor in concurrent visible and NIR-I windows. A Balb/c mouse was inoculated with 4T1 murine breast tumors on the right hindlimb was intravenously injected with IRDye800-TRC105. The surgery was performed 24 h post injection. Fluorescence was collected in the NIR-I (900-1000 nm) window excited by an 808-nm laser. The room light was used as illumination for color image. The handheld imager worked in the large-FOV mode.

Movie S2 (separate file). Molecular image-guided tumor resection in concurrent visible and NIR-I windows. Tumor removal was performed after the procedure shown in Supplementary Video 1.

Movie S3 (separate file). Molecular image-guided surgery of cutting the skin covering 4T1 tumor in concurrent visible and NIR-IIb windows. A Balb/c mouse bearing 4T1 murine breast tumors on the left hindlimb was intravenously injected with ErNPs-TRC105. The surgery was performed 24 h post injection. Fluorescence was collected in the NIR-IIb (1500-1700 nm) window excited by a 940-nm LED. The handheld imager worked in the large-FOV mode.

Movie S4 (separate file). Molecular image-guided tumor resection in concurrent visible and NIR-IIb windows. The skin containing tumor lesions was excised after the procedure shown in Supplementary Video 3.

Movie S5 (separate file). Molecular image-guided tumor resection in concurrent visible and NIR-IIb windows. The main tumor was excised after the procedure shown in Supplementary Video 4.

Movie S6 (separate file). High-resolution molecular image-guided tumor resection in concurrent visible and NIR-IIb windows. The small residual tumor was targeted by ErNPs-TRC105. This surgery was performed after the procedure shown in Supplementary Video 5. A 5X objective (NA = 0.12) was used in this observation.

SI References

1. Mai, H.-X. *et al.* High-Quality Sodium Rare-Earth Fluoride Nanocrystals: Controlled Synthesis and Optical Properties. *Journal of the American Chemical Society* **128**, 6426-6436 (2006).
2. Zhong, Y. *et al.* In vivo molecular imaging for immunotherapy using ultra-bright near-infrared-IIb rare-earth nanoparticles. *Nature Biotechnology* **37**, 1322-1331 (2019).