Supplementary Information

A near-infrared phosphorescent nanoprobe enables quantitative, longitudinal imaging of tumor hypoxia dynamics during radiotherapy

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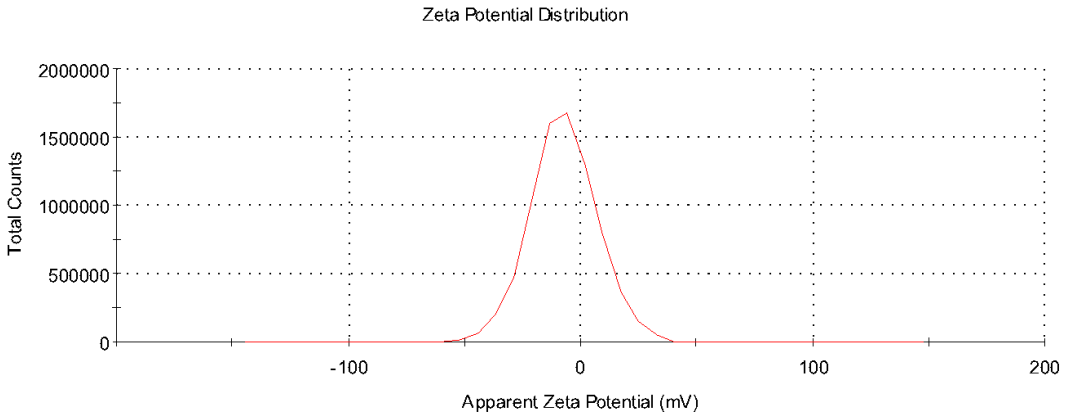
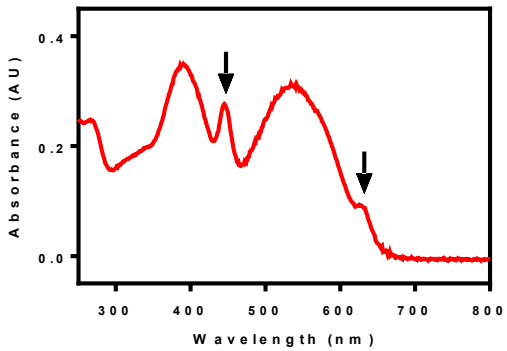
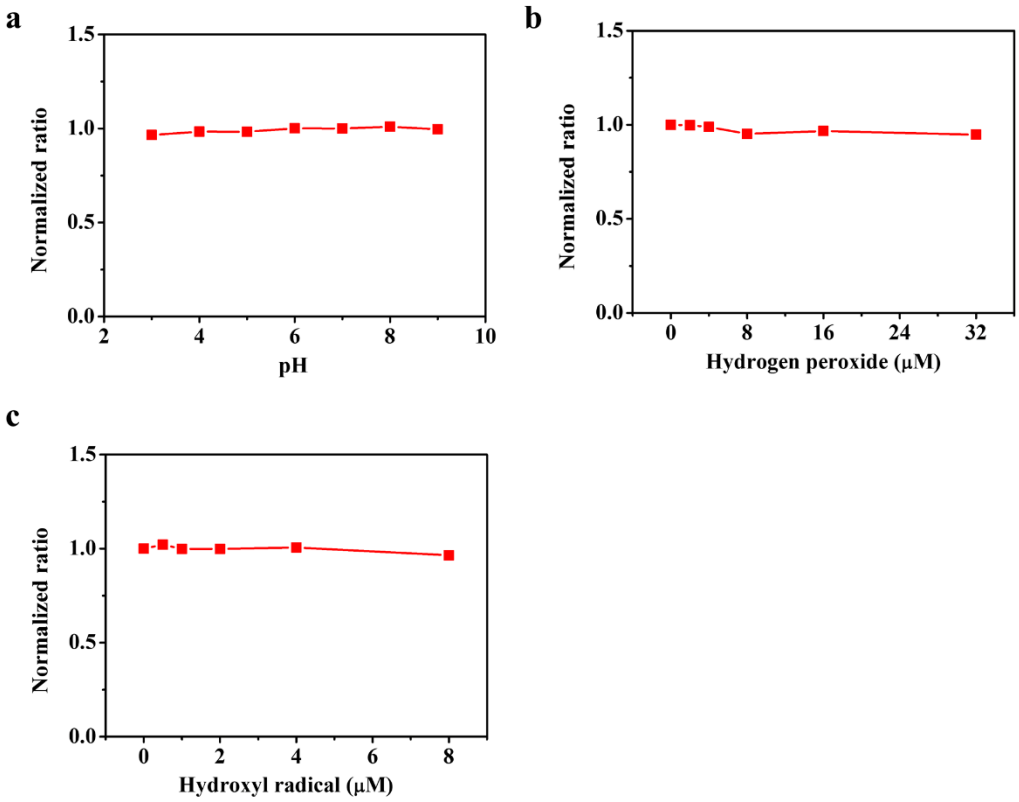
Supplementary Figures



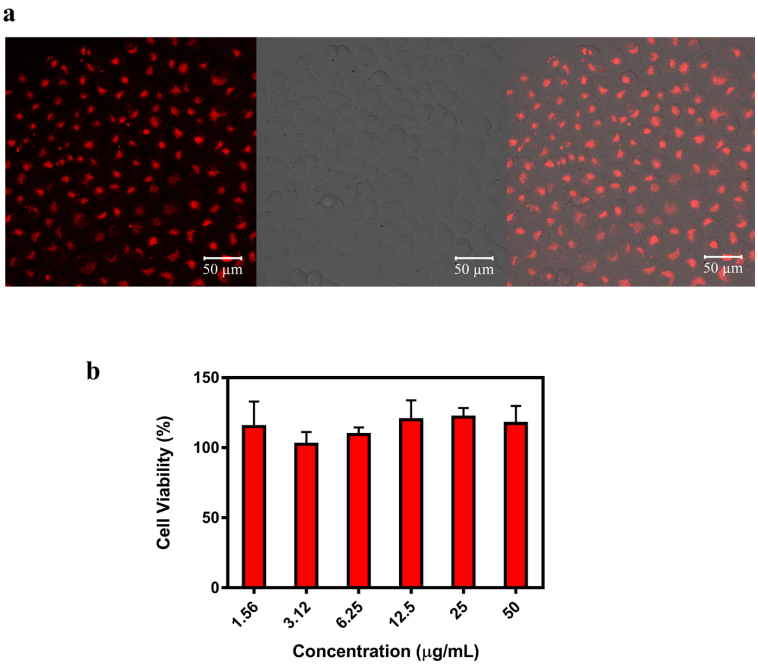
Supplementary Fig. 1. The zeta-potential distribution of the RHyLI nanoprobe in PBS (1×, pH 7.4). Peak value: -7 mV.



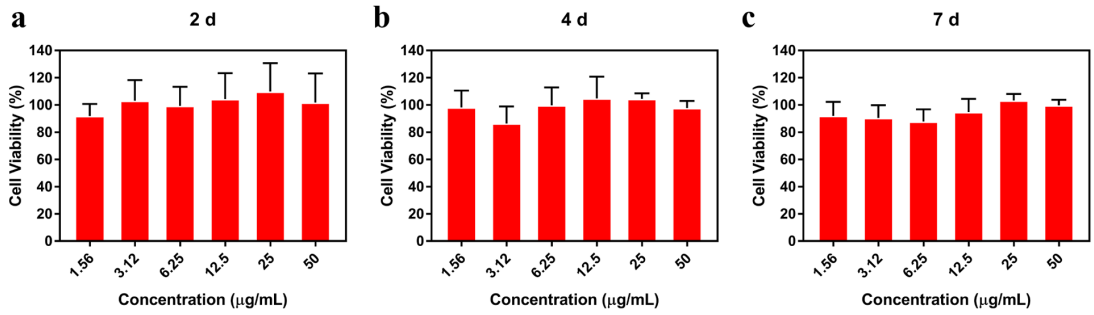
Supplementary Fig. 2. The absorption spectrum of the RHyLI nanoprobe in distilled water (20 μg/mL). Two black arrows indicate the absorption peaks from the embedded Pd complex (445 and 629 nm).



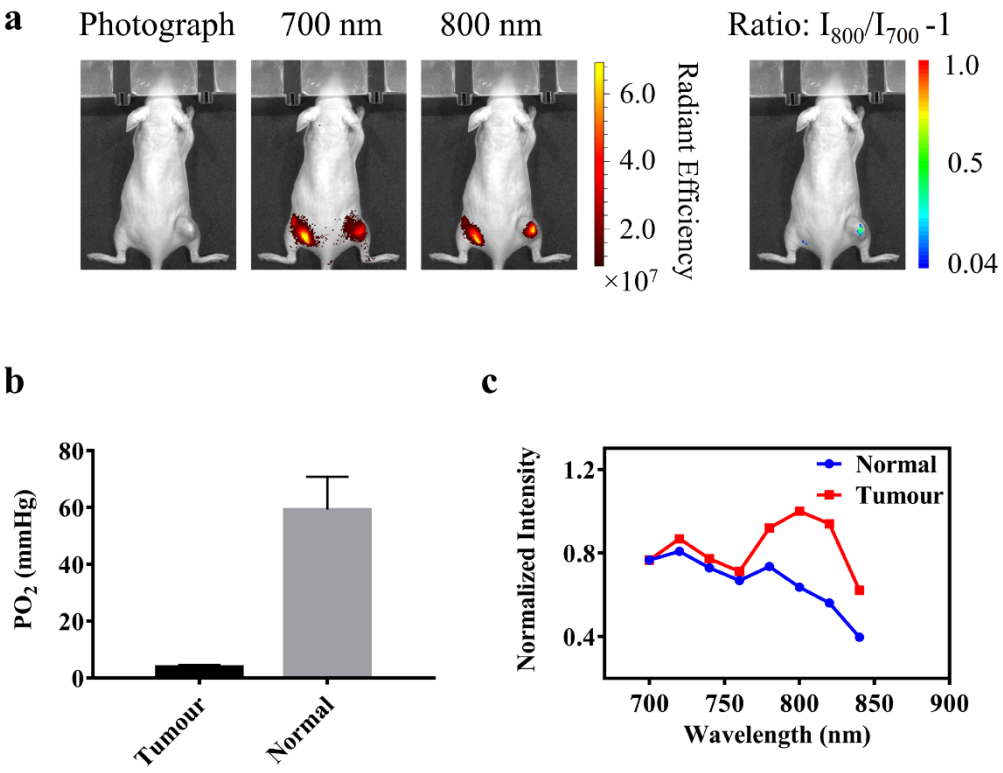
Supplementary Fig. 3. The response of the RHyLI nanoprobe to (a) pH (3 – 9), (b) hydrogen peroxide (0 – 32 μM), and (c) hydroxyl radical (0 – 8 μM). The change of the ratio ($R_{795/685}$) was within 5 % at high concentrations of hydrogen peroxide or hydroxyl radical.



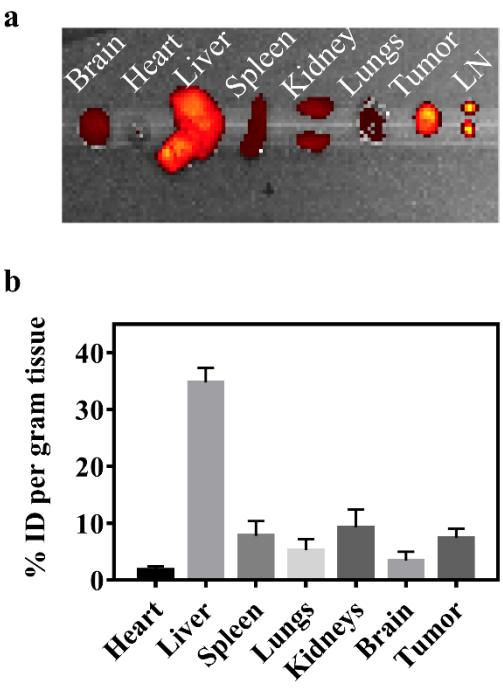
Supplementary Fig. 4. Cell uptake and cytotoxicity test of the nanoprobe. (a) Confocal fluorescence images of the HeLa cells after incubation with the nanoprobe (5 μg/mL) at 37 ºC for 2 h. Excitation: 561 nm; Emission: 650 – 750 nm. The cell uptake of the nanoprobe was observed by the red fluorescence signal in the cytoplasm. (b) HeLa cells were incubated with the nanoprobe at various concentrations (1.56–50 μg/mL) at 37 ºC for 24 h and the cell viabilities were tested using a standard MTT assay. No cytotoxicity was observed with the probe concentration up to 50 μg/mL. Results are Mean ± SD (N = 6).



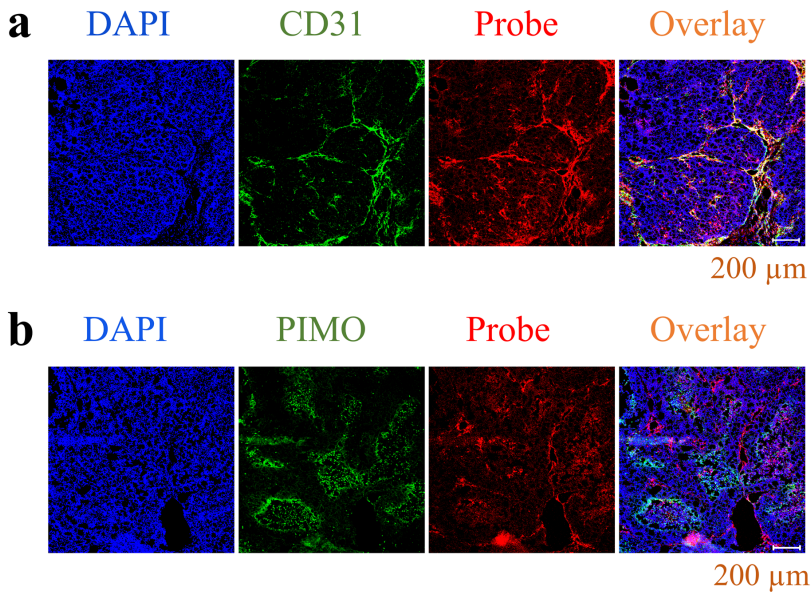
Supplementary Fig. 5. Cytotoxicity test of the nanoprobe with RWPE-1 cells. Normal human prostate epithelial RWPE-1 cells were incubated with the nanoprobe at various concentrations (1.56–50 μg/mL) at 37 ºC for (a) 2, (b) 4 or (c) 7 days. The culture media containing the corresponding concentrations of nanoprobe were refreshed every 2 days. The cell viabilities were tested using a standard MTT assay. No cytotoxicity of the nanoprobe to RWPE-1 cells was observed within 7 days with the probe concentration up to 50 μg/mL. Results are Mean ± SD (N = 6).



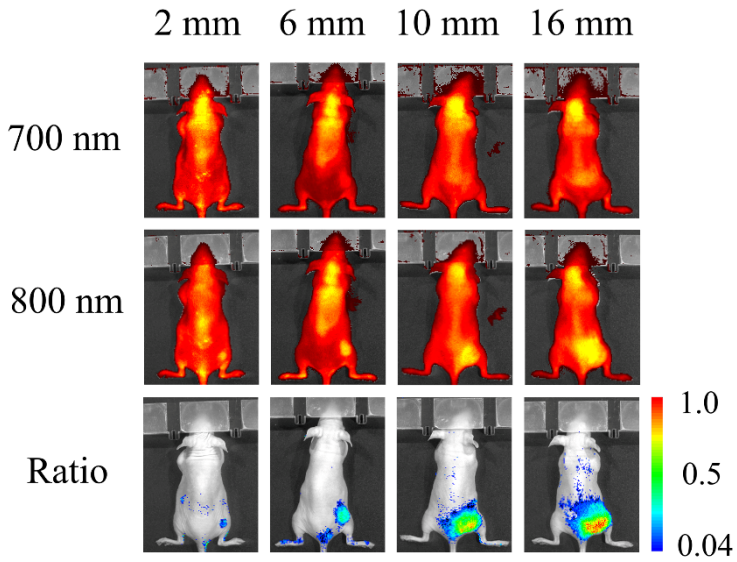
Supplementary Fig. 6. Validating the ratiometric response of the nanoprobe to the hypoxic condition of the tumors. (a) A nude mouse bearing HeLa tumor was imaged after intratumor injection of the nanoprobe in PBS (40 μL, 0.1 mg/mL) to the right tumor tissue and subcutaneous injection of the same amount of the nanoprobe to the left normal tissue. The photoluminescence signals were collected in two channels (700 and 800 nm) with the excitation at 640 nm and the ratiometric image of the two channels was obtained. (b) The oxygen partial pressure (PO_2) of the tumor tissue on the right thigh and the normal tissue on the left thigh of the nude mouse was measured using a needle-type oxygen microelectrode. Results are Mean ± SD (N = 25). (c) The emission spectra of the nanoprobe in the normal and tumor tissues.



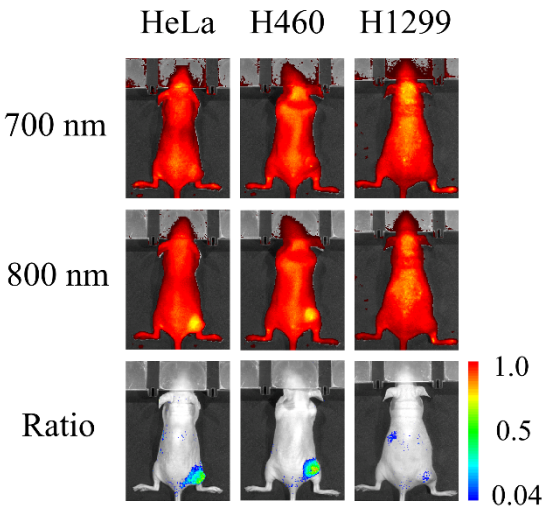
Supplementary Fig. 7. The bio-distribution of the RHyLI nanoprobe. Nude mice bearing HeLa tumor were i.v. injected with the nanoprobe (2 mg/kg). The probe distribution in various tissues and organs was (a) directly observed by *ex vivo* imaging in the 700 nm channel and (b) quantified using ICP-MS. Results are presented as Mean ± SD (N = 3).



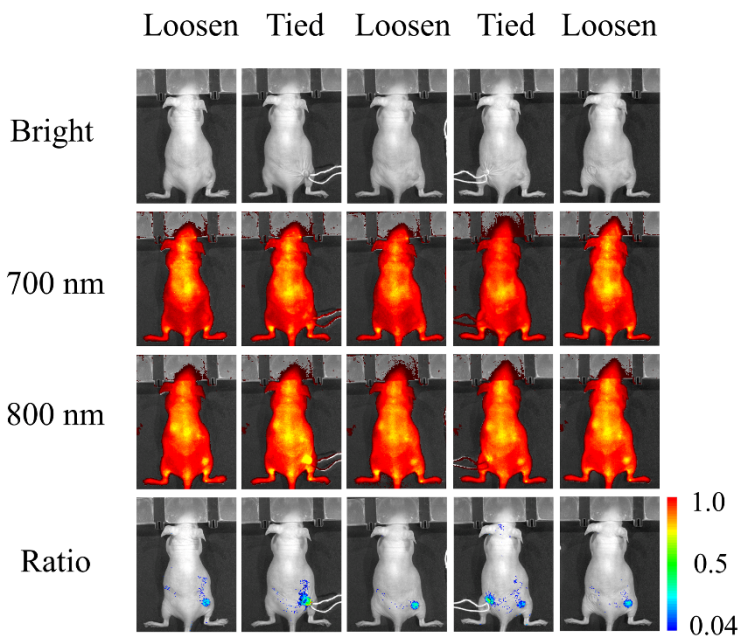
Supplementary Fig. 8. Immunofluorescence staining of the frozen sections of H460 tumors. The tumor tissues were collected at 24 h after i.v. injection of the nanoprobe (2 mg/kg). The blood vessels and hypoxic regions were revealed by (a) CD31 and (b) PIMO staining, respectively. The nanoprobe extravasation from the blood vessels and penetration into the hypoxic regions of the tumor tissue can be observed.



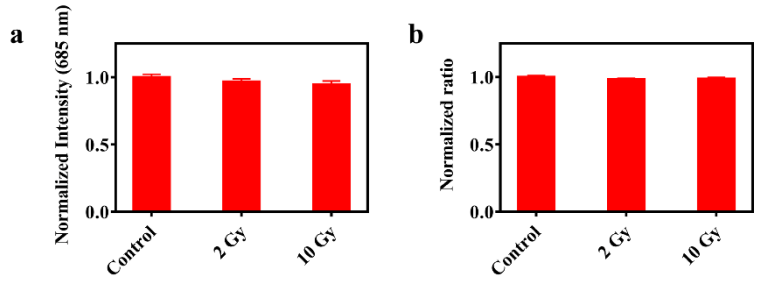
Supplementary Fig. 9. Nude mice bearing HeLa tumors with different tumor size (2, 6, 10, 16 mm, N = 3 for each group) were i.v. injected with the nanoprobe (2 mg/kg) and the ratiometric imaging was performed at 24 h p.i.



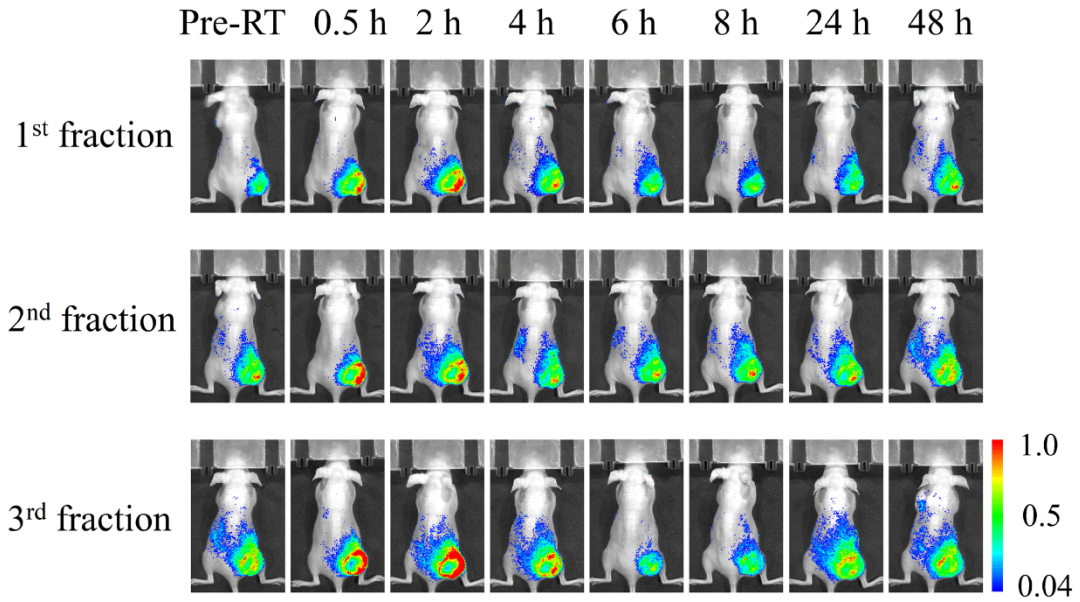
Supplementary Fig. 10. Nude mice bearing different tumor types (HeLa, H460, or H1299, size 8 mm, N = 3 for each group) were i.v. injected with the nanoprobe (2 mg/kg) and the ratiometric imaging was performed at 24 h p.i.



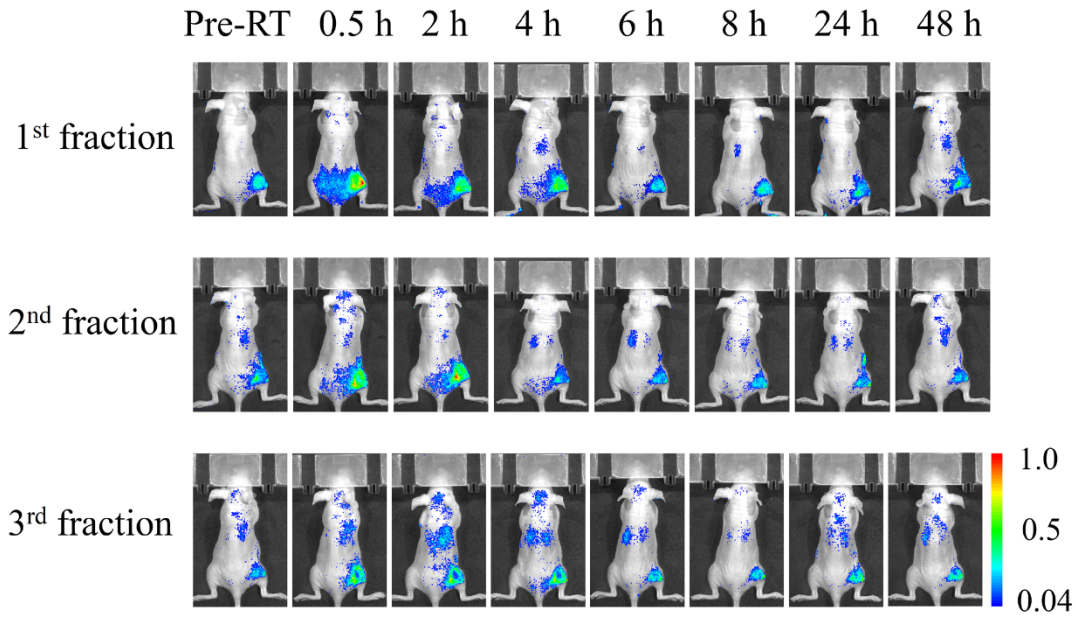
Supplementary Fig. 11. A nude mouse bearing HeLa tumor (2 mm) was i.v. injected with the nanoprobe (2 mg/kg). At 24 h p.i., the tumor tissue on the right thigh (red arrow) or the normal tissue on the left thigh (white arrow) was tied with a string to induce the temporal increase of hypoxia and then untied for the recovery of blood circulation. The change of hypoxia in this process was monitored by NIR ratiometric imaging.



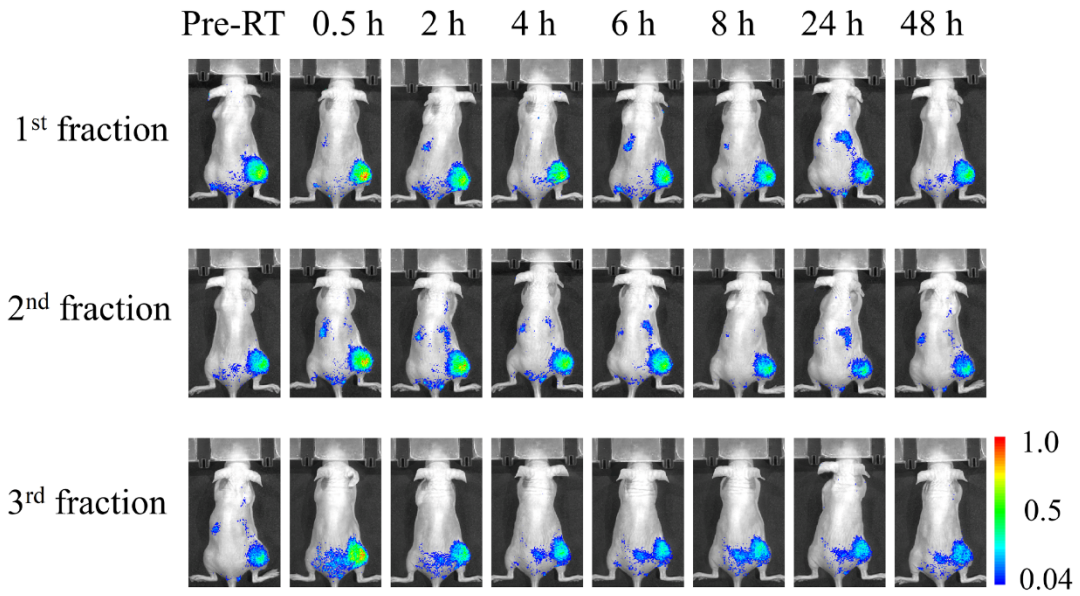
Supplementary Fig. 12. Stability of the nanoprobe upon radiation. PBS suspension of the nanoprobe (50 μg/mL, 50 uL) in a plastic tube was irradiated with a Kimtron Polaris MC-500 X-RAY irradiator at various doses (0, 2, and 10 Gy per fraction, 3 fractions). Then the samples were diluted with PBS to 1 mL and the emission spectra of the samples were collected with the excitation at 630 nm under air condition. The emission intensity at 685 nm (a) and the ratio between the emission intensities at 795 and 685 nm (b) were calculated and compared. Results are presented as Mean ± SD (N = 3). No significant change was observed in the photoluminescence properties of the nanoprobe at the tested radiation dose.



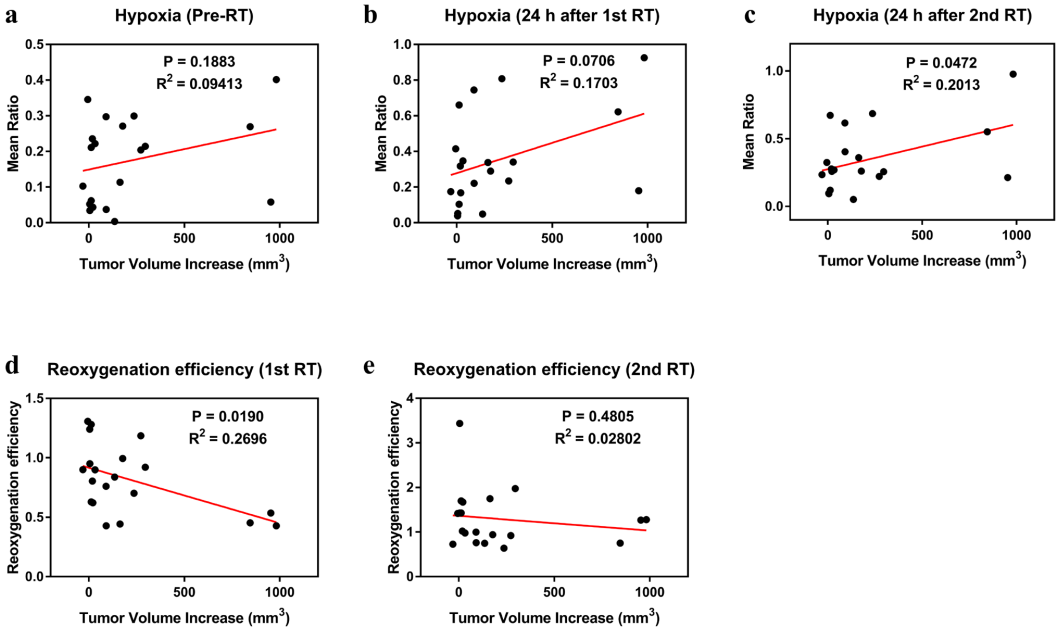
Supplementary Fig. 13. Ratiometric images of a mouse bearing HeLa tumor (8 mm) at various time points during the treatment of three low-dose RT fractions (2 Gy per fraction).



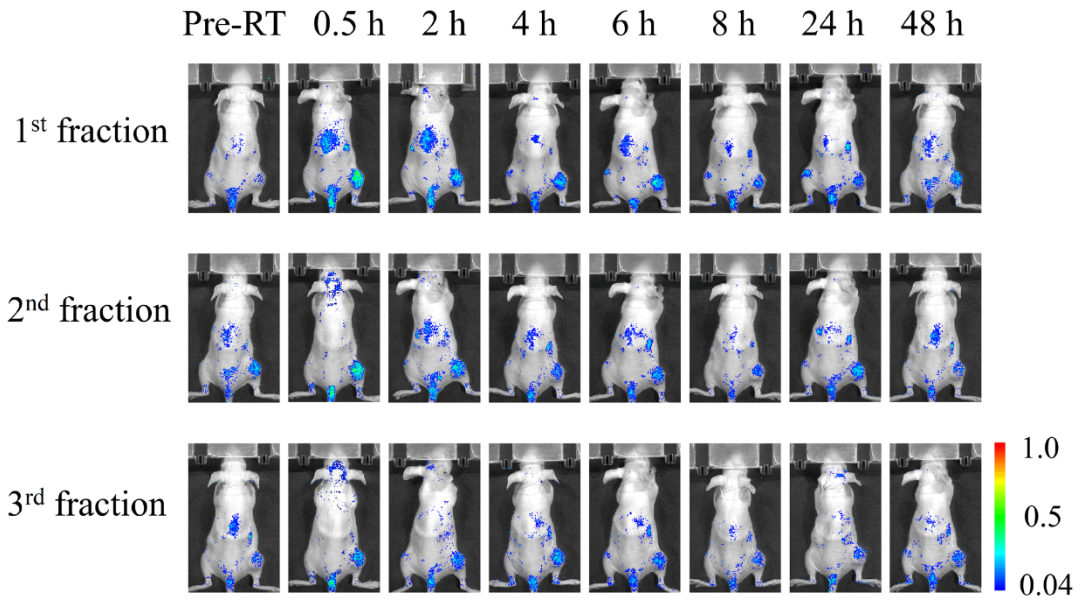
Supplementary Fig. 14. Ratiometric images of a mouse bearing HeLa tumor with smaller size (5 mm) at various time points during the treatment of three RT fractions (10 Gy per fraction).



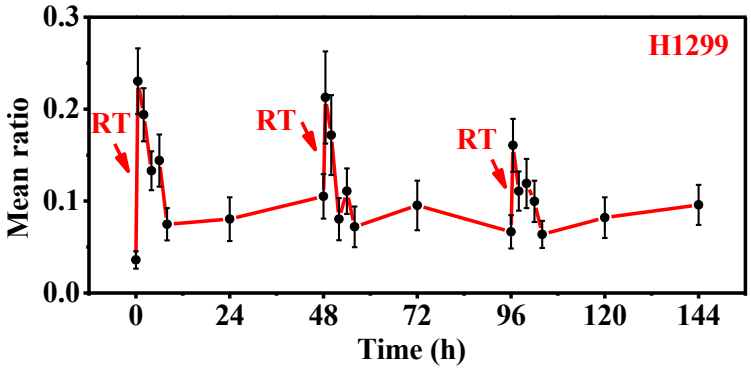
Supplementary Fig. 15. Ratiometric images of a mouse bearing H460 tumor (8 mm) at various time points during the treatment of three RT fractions (10 Gy per fraction).



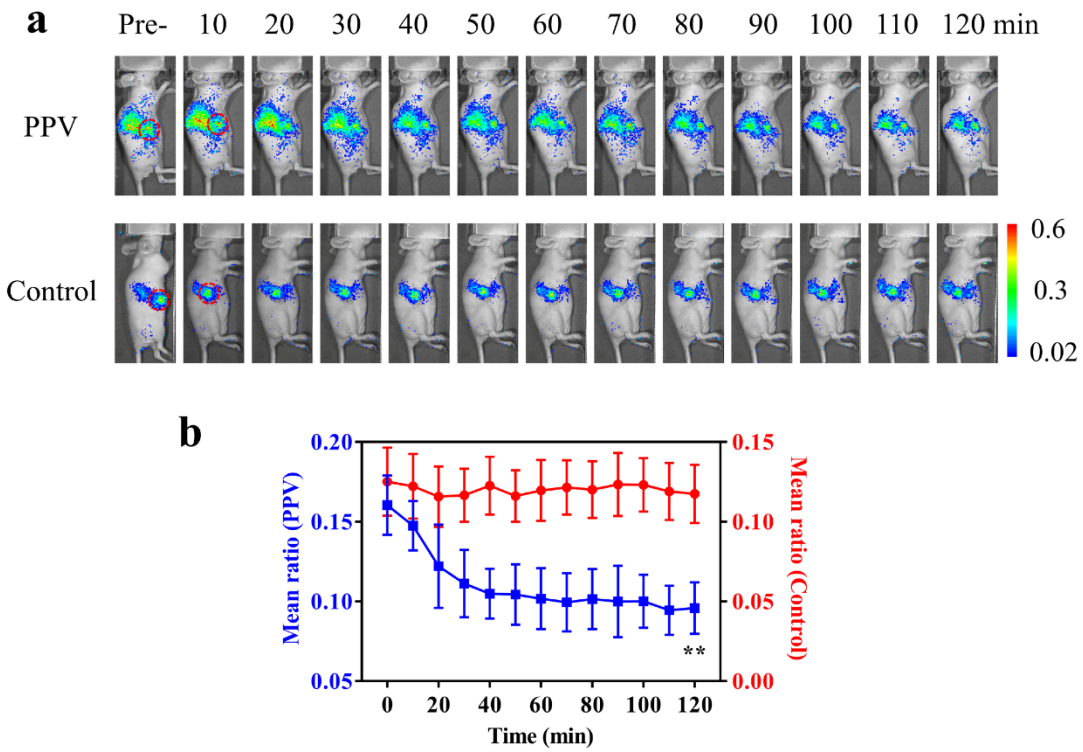
Supplementary Fig. 16. Correlation between tumor volume increase and tumor hypoxia. The increase of tumor volume from day 0 to day 15 was correlated with the hypoxia levels (a) before RT, (b) at 24 h after the 1st fraction, and (c) at 24 h after the 2nd fraction, as well as the reoxygenation efficiency in the (d) 1st fraction and (e) 2nd fraction. The data of tumor volume increase and tumor hypoxia of the 20 mice in group 1 and 2 (Fig. 5F-H) were analyzed. Two-tailed P values are demonstrated (confidence interval 95%, assume Gaussian distribution).



Supplementary Fig. 17. Ratiometric images of a mouse bearing H1299 tumor (8 mm) at various time points during the treatment of three RT fractions (10 Gy per fraction).



Supplementary Fig. 18. Evolution of tumor hypoxia over time when nude mice bearing H1299 tumors (8 mm) were treated with three RT fractions (10 Gy per fraction). Results are Mean ± SD (N = 3).



Supplementary Fig. 19. Monitoring the response of tumor hypoxia upon drug treatment. (a) Nu/Nu nude mice bearing subcutaneous A549 tumor were treated with papaverine hydrochloride (PPV, 2 mg/kg) or PBS by i.v. injection. The evolution of tumor hypoxia upon drug treatment was monitored by ratiometric imaging with the RHyLI nanoprobe. The red circles indicate the tumor locations. (b) The values of mean ratio (representing tumor hypoxia) at each time point after drug treatment were plotted. Results are presented as Mean ± SD (N = 3). ** $p < 0.01$, in comparison between the mean ratio values at 0 and 120 min after PPV treatment by two-tailed t-test with Welch's correction.

Supplementary Methods

Materials. The semiconducting polymer, Poly[2,7-(9,9-dioctylfluorene)-alt-4,7-bis(thiophen-2-yl)benzo-2,1,3-thiadiazole] (PFO-DBT, M_w 10,000 – 50,000) was purchased from Millipore Sigma. Meso-Tetraphenyl-tetrabenzoporphine Palladium (Pd-TPTBP) was purchased from Fisher Scientific. Poly(styrene-b-ethylene oxide) (Mn 1,600-b-2,500, PDI 1.08) was purchased from Polymer Source (P2972-SEO).

General instruments. TEM images were captured on a JEOL Transmission Electron Microscope (JEM-1400). Hydrodynamic diameter and zeta potential were measured on a Zetasizer Nano ZS90 dynamic light scattering (DLS) analyzer (Malvern Instruments). The absorption spectra were collected on an Agilent Cary 8453 UV-Visible Spectrophotometer. The emission spectra were collected on a Horiba Jobin Yvon FluoroMax-3 spectrofluorometer. Immunofluorescence images were captured on a Zeiss LSM 880 laser scanning confocal microscope. The concentration of palladium (Pd) was determined with a high-resolution inductively coupled plasma mass spectrometer (ICP-MS, NU ATTOM)). Ratiometric imaging of tumor hypoxia was performed on an IVIS spectrum *in vivo* imaging system (PerkinElmer). The oxygen partial pressure of the mice tissues was measured with a needle-type oxygen microelectrode (Unisense, OX-100). Radiotherapy of the tumor-bearing mice was performed on a Kimtron Polaris MC-500 X-ray irradiator.

Cell uptake and cytotoxicity test. To test the cell uptake of the nanoprobe, HeLa cells were incubated with the RHyLI nanoprobe (5 μg/mL) at 37 ºC for 2 h in 35 mm glass bottom dishes and then washed with PBS (1x) for three times. The confocal fluorescence images of the cells were collected on the Zeiss LSM 880 microscope with the excitation at 561 nm and emission at 650 – 750 nm. To test the cytotoxicity of the RHyLI nanoprobe, HeLa cells were incubated with the nanoprobe at various concentrations (1.56, 3.12, 6.25, 12.5, 25, and 50 μg/mL) at 37 ºC for 24 h in the 96-well cell culture plates. Then MTT (5 mg/mL, 20 μL) was added to each well followed with another 3 h incubation at 37 °C. The cell culture medium was replaced with DMSO (200 μL for each well) and the 96-well cell culture plates were gently shaken for 10 min at room temperature to dissolve the formed compound. The absorbance of each well at 570 nm was recorded on a microplate reader (Tecan Safire). The cell viability was calculated by the ratio of the absorbance of the cells incubated with the nanoprobe to that of cells incubated with culture medium only.

Immunofluorescence staining. Nude mice bearing HeLa tumor on the right rear thigh were i.v. injected with the RHyLI nanoprobe in 100 μL PBS (2 mg/kg). At 24 h p.i., pimonidazole hydrochloride in 100 μL PBS was i.v. injected (60 mg/kg) and the mice were sacrificed 1 h later. The tumor tissues were collected, fixed with 4% paraformaldehyde overnight, placed in 15% sucrose in PBS until the tumor tissues sank. The sucrose drop was repeated in 30% sucrose in PBS. Then the tumor tissues were embedded in optimal cutting temperature (OCT) compound and sectioned in a

freezing microtome.

The frozen sections were incubated with TX-100 (0.2% in PBS) for 15 min at room temperature, washed with PBS for three times, and blocked with 1% BSA for 30 min at room temperature. Then the frozen sections were added with the diluted primary antibody, incubated at 4 ºC overnight, washed three times with PBS, added with the diluted secondary antibody, incubated at room temperature for 1 h in dark, washed three times with PBS, stained with DAPI (1 μg/mL) at room temperature for 5 min in dark, followed by three washes with PBS. For CD31 staining, the primary antibody was a rabbit polyclonal anti-CD31 antibody (Abcam, ab28364, 200× dilution with 1% BSA) and the secondary antibody was a goat anti-rabbit IgG H&L antibody labelled with AF488 (Abcam, ab150077, 300× dilution with 1% BSA). For hypoxia staining, the primary antibody was mouse IgG1 monoclonal antibody to pimonidazole (PIMO) labelled with FITC (FITC-MAb1, Hypoxyprobe^{TM} Kit, 100× dilution with 1% BSA). No secondary antibody was used.

The tissue sections were imaged on a Zeiss LSM 880 laser scanning confocal microscope. The DAPI staining was detected in the blue channel (430–500 nm) with the excitation at 405 nm. The CD31 or PIMO staining was detected in the green channel (510–580 nm) with the excitation at 488 nm. The probe signal was detected in the red channel (650–750 nm) with the excitation at 561 nm. The obtained images were imported into Image-Pro Plus (v6.0.0, Media Cybernetics), where the signal intensities of the probe, CD31, and PIMO staining were analyzed in three different regions of the whole tumor sections, including periphery, semi-periphery, and the

center. The results were presented as the signal intensities per area.

The immunofluorescence staining of the frozen sections of H460 tumors was performed using the same protocol as mentioned above.

Testing the probe response to tumor hypoxia by intratumor injection. A nude mouse bearing HeLa tumor was intratumorly injected with the RHyLI nanoprobe in PBS (40 μL, 0.1 mg/mL) to the right tumor tissue. For comparison, the same amount of the RHyLI nanoprobe was subcutaneously injected to the left normal tissue. Then the mouse was imaged in IVIS spectrum *in vivo* imaging system. For ratiometric imaging, the photoluminescence signals were collected in two channels: 700 nm (690–710 nm filter) and 800 nm (790–810 nm filter), with the excitation at 640 nm (620–660 nm filter). To obtain the emission spectra of the tumor and normal tissues, the mouse was excited at 640 nm and the photoluminescence signals were collected in eight channels: 700, 720, 740, 760, 780, 800, 820, and 840 nm. The signal intensities obtained in these channels were calculated for the tumor and normal tissues to generate the emission spectra.

Longitudinal imaging of tumor hypoxia. NU/NU nude mice bearing HeLa tumor (6 mm) on the right rear thigh (N = 3) were i.v. injected with the RHyLI nanoprobe in 100 μL PBS (2 mg/kg). The tumor-bearing mice were imaged by ratiometric imaging at various time points (1, 4, 8, 24, 48 h, and 7 d) p.i..

Hypoxia imaging at different tumor sizes and types. To test the hypoxia imaging at different tumor sizes or tumor types, nude mice bearing HeLa tumors at a size of 2, 6, 10, or 16 mm, or 8 mm HeLa, H460, or H1299 tumors on the right rear thigh (N = 3 per group) were i.v. injected with the RHyLI nanoprobe in 100 μL PBS (2 mg/kg). The tumor-bearing mice were imaged by ratiometric imaging at 24 h p.i. of the nanoprobe.

Reversibility of the probe response to tumor hypoxia. To test the reversibility of the probe response to tumor hypoxia, nude mice bearing HeLa tumor (2 mm) on the right rear thigh (N = 3) were i.v. injected with the RHyLI nanoprobe in 100 μL PBS (2 mg/kg). At 24 h p.i., the tumor-bearing mice were imaged by ratiometric imaging. Then a string was used to tie the tumor tissues for 3 min and the mice were imaged again. Afterwards, the string was untied, followed with another ratiometric imaging 3 min later. To test the reversible response of the nanoprobe in the normal tissues, a string was used to tie and then loosen the normal tissues on the left rear thigh of the mice with the same protocol, and the change of hypoxia in the normal tissues was observed by ratiometric imaging.

Biodistribution study. Nude mice bearing HeLa tumor on the right rear thigh (N =3) were i.v. injected with the RHyLI nanoprobe in 100 μL PBS (2 mg/kg). At 24 h p.i., the tumor-bearing mice were sacrificed to collect the tumor tissues and various normal organs. The distribution of the nanoprobe in these tissues and organs was

directly observed by *ex vivo* imaging in the 700 nm channel in the IVIS spectrum *in vivo* imaging system. To quantify the probe distribution, the tissues and organs were weighted and digested in concentrated nitric acid. The concentrations of Pd in these samples were determined using ICP-MS. The Pd concentrations of the tissues and organs from tumor-bearing mice i.v. injected with PBS only served as blank reference. The bio-distribution was presented as the percentage of the nanoprobe in each tissue or organ in the total injection dose, divided by the weight of the corresponding tissue or organ (% ID per gram tissue).

Monitoring the response of tumor hypoxia upon drug treatment. Nu/Nu nude mice bearing subcutaneous A549 tumor (N = 10, tumor size: 6-8 mm in diamater) were i.v. injected with RHyLI nanoprobe (2 mg/kg). As shown in the timeline below, at 24 h post-injection, the levels of tumor hypoxia were measured by ratiometric imaging. Six mice with relatively high tumor hypoxia levels were selected for further treatment and randomly distributed to two groups (N = 3 for each group). At 48 h after injection of the nanoprobe, the two groups of tumor-bearing mice were treated with Papaverine hydrochloride (PPV, 2 mg/kg) or PBS by a single i.v. injection. The levels of tumor hypoxia were continuously monitored by ratiometric imaging before and after treatment for 2 hrs with a time interval of 10 min.