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Supporting Information

Repeatable deep-tissue activation of persistent luminescence nanoparticles by soft X-ray for high sensitivity long-term *in vivo* bioimaging

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Materials and method

Chemicals. Water soluble CdSe quantum dots were purchased from Xingzi (Shanghai) New-material technology development Co. Ltd (China). 3-amino propyltriethoxysilane (APTES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), strontium carbonate ($SrCO_3$, 99.9%), aluminum oxide (Al_2O_3, 99.9%), europium oxide (Eu_2O_3, 99.9%) were purchased from Sigma-Aldrich. Alpha-methoxy-omega-carboxylic acid poly(ethylene glycol) (MeO-PEG-COOH, 3000 Da) was purchased from Xi'an Ruixi Biological Technology Co. Ltd. (China). All other chemicals were of analytical grade and were used as received from manufacturer. Ultrapure water obtained from a Millipore water purification system (18.2 MΩ resistivity) was used in all runs.

Apparatus. Ultraviolet-visible-near-infrared light (UV-Vis-NIR) absorption spectra were recorded using a SH-1000 Lab microplate reader (Corona Electric, Hitachinaka, Japan) at room temperature. Transmission electron microscopy (TEM) images were taken on the FEI Tecnai G20 transmission electron microscope with an accelerating voltage of 200 kV. X-ray diffraction (XRD) patterns were obtained by using an X-ray powder diffractometer (D/MAX-3C, Rigaku Co., Japan). Photoluminescence (PL) measurements were performed using a Edinburgh FS5 fluorescence spectrophotometer (Edinburgh Instruments Ltd., UK). X-rays generated from a mini-X X-ray tube (Amptek, Inc.). Luminescence and X-ray-

induced luminescence imaging were performed using a SI Imaging Amix small animal imaging system (Spectral Instruments Imaging Co., USA).

Preparation of $SrAl_2O_4:Eu^{2+}$ persistent luminescence powder. 3.45 mmol $SrCO_3$, 3.45 mmol Al_2O_3, 0.45 mmol Eu_2O_3 were thoroughly mixed with minimum ethanol and ground for 1 h. The mixture was calcined at 900 ^oC for 1 h in air. Then, the mixture was sintered at 1300 ^oC for 4 h in a reducing atmosphere (5% H_2 + 95% Ar).

Surface functionalization of $SrAl_2O_4:Eu^{2+}$. The $SrAl_2O_4:Eu^{2+}$ powder was wet ground in minimum ethanol for 4 h. The obtained sample was dispersed in 5 mM NaOH ethanol solution by sonication for 3 h, and then vigorously stirred for 12 h. The resulting colloid solution was centrifuged at 2000 rpm for 5 min to remove large size particles, and the supernatant was centrifuged at 8000 rpm for 7 min to collect the precipitate. A 5 mg sample of dry precipitate was redispersed in 2 mL of dimethylformamide (DMF) by sonication, and 50 µL of APTES was added under vigorous stirring at 80 ^oC for 12 h. The resulting APTES-$SrAl_2O_4:Eu^{2+}$ were collected by centrifugation, and washed with DMF to remove unreacted APTES.

5 mg of APTES-$SrAl_2O_4:Eu^{2+}$ nanoparticles were dispersed in 10 mM PBS (pH 6), EDC (4 mg) and NHS (10 mg) were addd. The mixture was gently stirred at room temperature for 2 h. Following activation, 35 mg of MeO-PEG-COOH was added, and the mixture solution was adjusted to pH 8 with NaOH solution and stirred in the dark at room

temperature for 8 h. The unreacted PEG was removed by centrifugation, and the resulting PEG-$SrAl_2O_4$:Eu^{2+} nanoparticles were washed three times with 10 mM PBS.

Cell culture and cytotoxicity assay. HepG2 cells were cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum (FBS, Gibco) at 37 ^{o}C in a humidified atmosphere with 5% CO_2. Cell viability was measured with CCK-8 according to the manufacturer's protocol. In a typical experiment, HepG2 cells were seeded in 96-well plates and then incubated with 100 μL of varying concentrations of PEG-$SrAl_2O_4$:Eu^{2+} nanoparticles for 24 h at 37 ^{o}C in a humidified 5% CO_2 atmosphere. Cell viabilities were determined by CCK-8 according to the manufacturer's protocol.

Biological safety of soft X-ray assay. HepG2 cells were cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum (FBS, Gibco) at 37 ^{o}C in a humidified atmosphere with 5% CO_2. Cell viability was measured with CCK-8 according to the manufacturer's protocol. In a typical experiment, HepG2 cells were seeded in 96-well plates and incubated with soft X-ray irradiation for 5 min under different X-ray tube voltage, the distance between X-ray tube and cells was about 10 cm, then HepG2 cells incubated for 24 h at 37 ^{o}C in a humidified 5% CO_2 atmosphere. Cell viabilities were determined by CCK-8 according to the manufacturer's protocol.

Simulation experiment of deep tissue penetration. The experiment was performed by placing a piece of pork (~2.5 cm) between the soft X-ray light source and the solution

of PEG-$SrAl_2O_4$:Eu^{2+} PLNPs. The PEG-$SrAl_2O_4$:Eu^{2+} PLNPs were activated by soft X-ray under dark field with pork as an X-ray blocker, X-ray tube was operated with a tube voltage of 40 kV and a tube current of 70 μA. The photographs were obtained using a Nikon digital camera.

***In vivo* soft X-ray-activated persistent luminescence optical imaging.** All experiments involving animals were approved by the Animal Ethics Committee of Fujian Medical University. BALB/c nude mice (weight ~20 g) were obtained from Shanghai SLAC laboratory Animal Co., Ltd. Tumor-bearing mice were prepared by subcutaneously injecting a suspension of 2×10^6 HepG2 cells in PBS (100 μL) into the back of the hind leg.

In vivo soft X-ray-activated persistent luminescence optical imaging was performed with SI Imaging Amix small animal imaging system. PEG-$SrAl_2O_4$:Eu^{2+} nanoparticles (2 mg mL^{-1}) dispersed in 10 mM PBS solution were injected through intratumoral injection into HepG2 tumor-bearing mice. The PEG-$SrAl_2O_4$:Eu^{2+} nanoparticles were first activated under soft X-ray irradiation (40 kV, 70 μA) for 90 s and then intratumoral injection immediately (100 μL, 2 mg mL^{-1} per mouse), the persistent luminescence signal was recorded at 2 min, and 1 h after injection, and at 2 h after intratumoral injection, persistent luminescence was reactivated following a 90 s soft X-ray excitation (40 kV, 70 μA), and immediately acquired the luminescence signal.

We provide *in vivo* whole body imaging by intravenous injection of PEG-$SrAl_2O_4:Eu^{2+}$ PLNPs into the BALB/c nude mice. The PEG-$SrAl_2O_4:Eu^{2+}$ nanoparticles were first activated under soft X-ray irradiation (40 kV, 70 μA) for 90 s and then intravenous injection immediately (200 μL, 2 mg mL^{-1} per mouse), the persistent luminescence signal was recorded at 5 min, 10 min, 30 min 60 min, 90 min and 120 min after injection, and at 2 h and 8 h after intravenous injection, persistent luminescence was reactivated following a 90 s soft X-ray excitation (40 kV, 70 μA), and immediately acquired the luminescence signal.

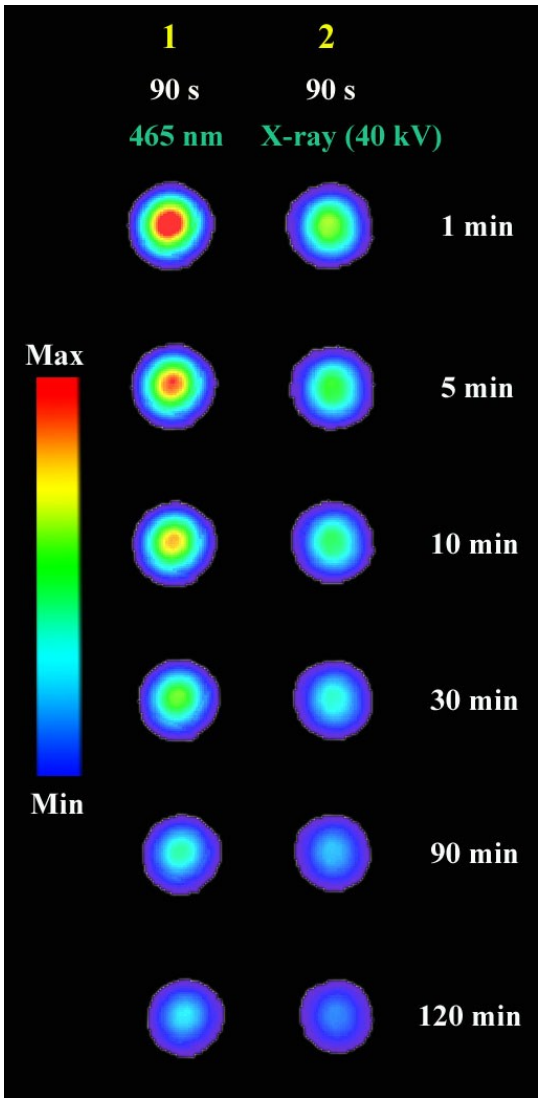


Fig. S1 Persistent luminescence images of $SrAl_2O_4:Eu^{2+}$ phosphor taken at different afterglow time (1 min to 120 min) after irradiation by 465 nm light source and soft X-ray (40 kV) for 90 s.

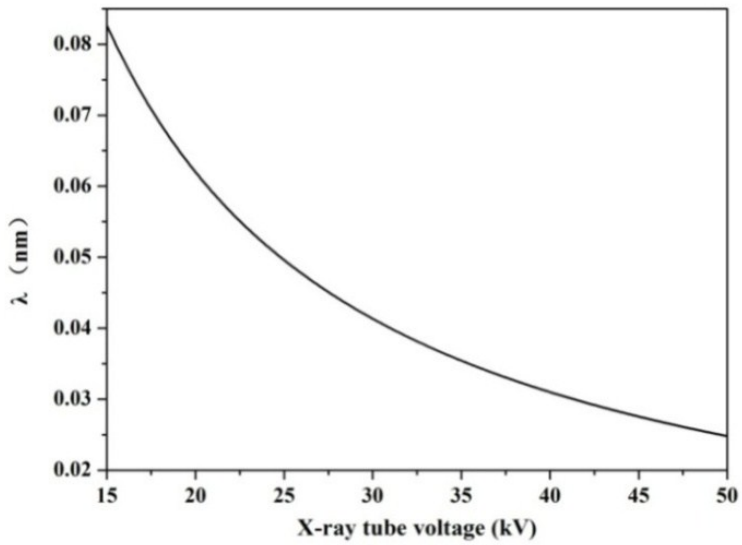


Fig. S2 The relationship between X-ray tube voltage and X-ray short-wavelength limit (λ_{SWT}).

The equation between X-ray tube voltage and X-ray short-wavelength limit was provided below.[1]

$$eV = hv = \frac{hc}{\lambda_{\min}} \qquad \lambda_{\min} = \lambda_{SWT} = \frac{hc}{eV} = \frac{12.398}{V} \text{ Å}$$

where: λ_{min} or λ_{SWT} is the X-ray short-wavelength limit (Å), 1 Å=0.1 nm; e=1.602×10^{-19} C;

h is the Planck constant (6.626×10^{-34} J s);

c is the Light speed (3×10^{8} m s^{-1});

V is the X-ray tube voltage (kV).

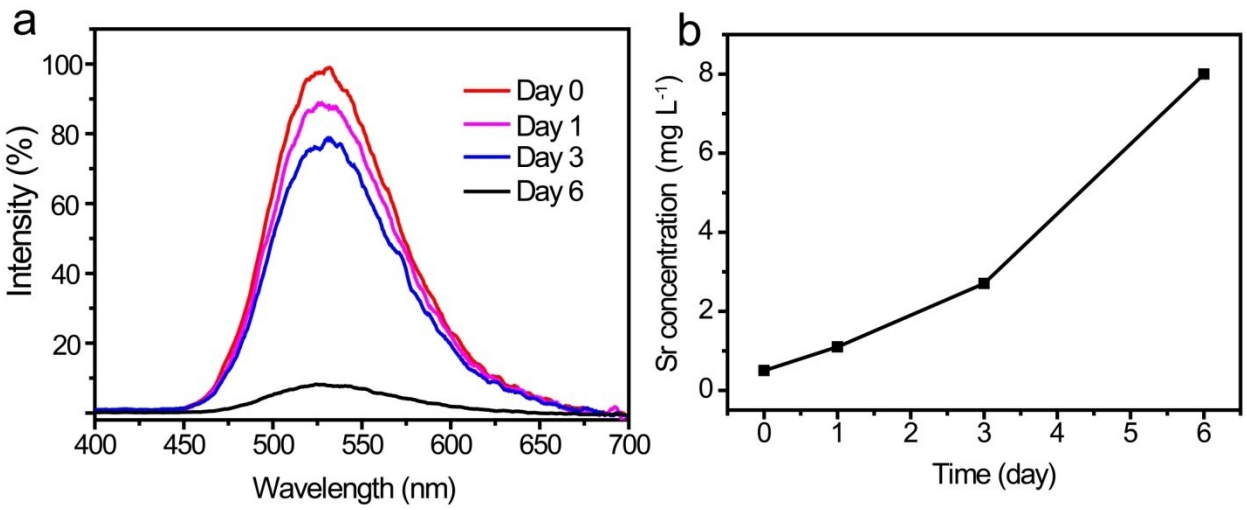


Fig. S3 (a) Change of photoluminescence intensity (soft X-ray excitation). There was a gradual drop of the PL intensity over time. (b) Quantification of strontium (Sr) concentration in supernatant solution after centrifugation of $SrAl_2O_4:Eu^{2+}$ PLNPs aqueous solutions by ICP-MS elemental analysis.

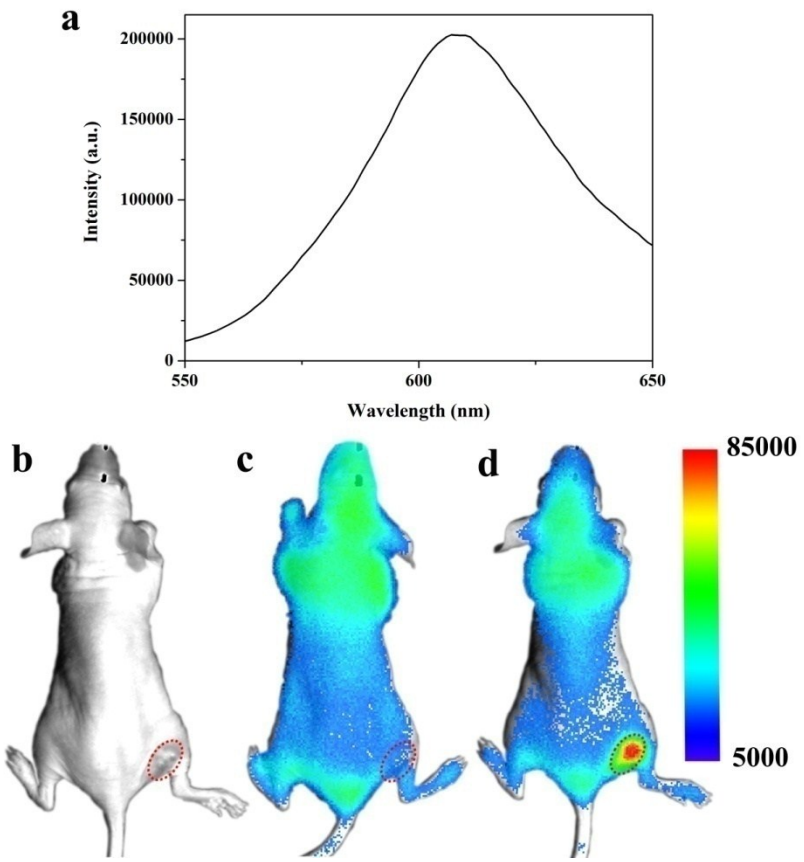


Fig. S4 *In vivo* optical imaging of CdSe quantum dots in HepG2 tumor-bearing mice (red circles locate the tumor site). a) Fluorescence spectra of CdSe quantum dots with emission peaks at ~608 nm excited by 465 nm light source. b) The bright field image before the CdSe quantum dots injected into mouse. c) Optical fluorescence image of HepG2 tumor-bearing mice excited by 465 nm light source before the intratumoral injection of CdSe quantum dots. d) Optical fluorescence image of HepG2 tumor-bearing mice excited by 465 nm light source after the intratumoral injection of CdSe quantum dots. The fluorescence intensity is expressed in false colour units (1 units=1000 photons s^{-1} cm^2 sr).

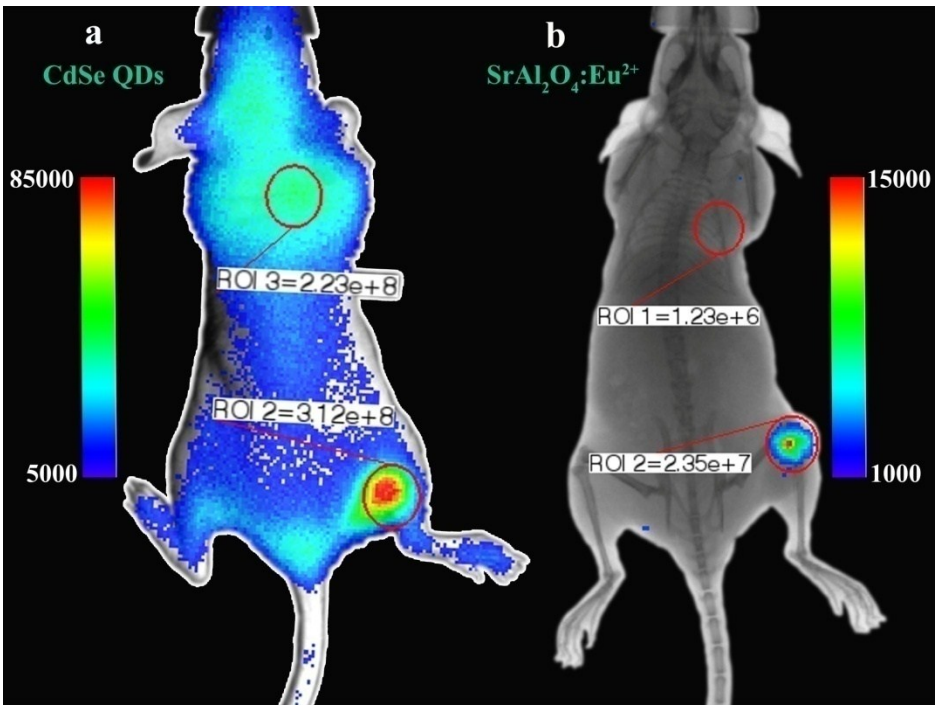
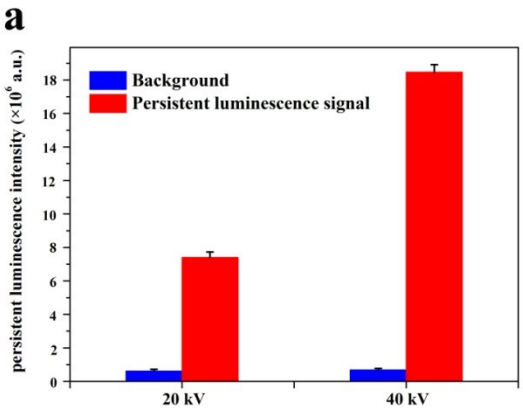
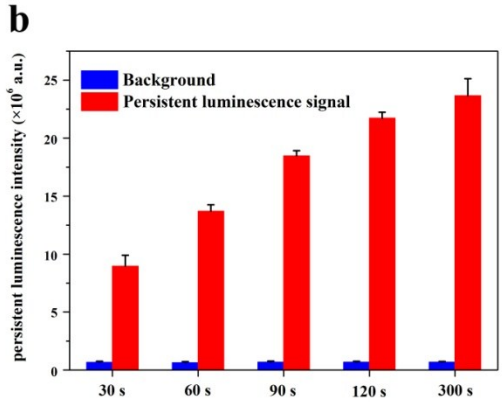


Fig. S5 *In vivo* imaging comparison of CdSe quantum dots with PEG-$SrAl_2O_4:Eu^{2+}$ nanoparticles. a) Optical fluorescence image of HepG2 tumor-bearing mice excited by 465 nm light source after the intratumoral injection of CdSe quantum dots. b) Persistent luminescence image of HepG2 tumor-bearing mice activated by soft X-ray irradiation after the intratumoral injection of PEG-$SrAl_2O_4:Eu^{2+}$ nanoparticles. The experiments were conducted by injecting the same amount (100 µL, 2 mg mL^{-1} per mouse) of CdSe quantum dots and PEG-$SrAl_2O_4:Eu^{2+}$ nanoparticles. The persistent luminescence or fluorescence intensity is expressed in false colour units (1 units=1000 photons s^{-1} cm^2 sr).



	20 kV	40 kV
Persistent luminescence signal ($\times 10^6$)	7.41	18.5
Background ($\times 10^6$)	0.618	0.683
Signal to background ratio	11.98	27.09



	30 s	60 s	90 s	120 s	300 s
Persistent luminescence signal ($\times 10^6$)	8.96	13.7	18.5	21.7	23.7
Background ($\times 10^6$)	0.671	0.634	0.683	0.686	0.684
Signal to background ratio	13.35	21.61	27.09	31.63	34.65

Fig. S6 The *in vivo* persistent luminescence signal intensity and signal-to-background ratio of PEG-$SrAl_2O_4$:Eu^{2+} PLNPs. a) Persistent luminescence was reactivated following 90 s soft X-ray activation with different tube voltage. b) Persistent luminescence was reactivated following different soft X-ray activation time (40 kV tube voltage). The error bars represent standard errors (n=3).

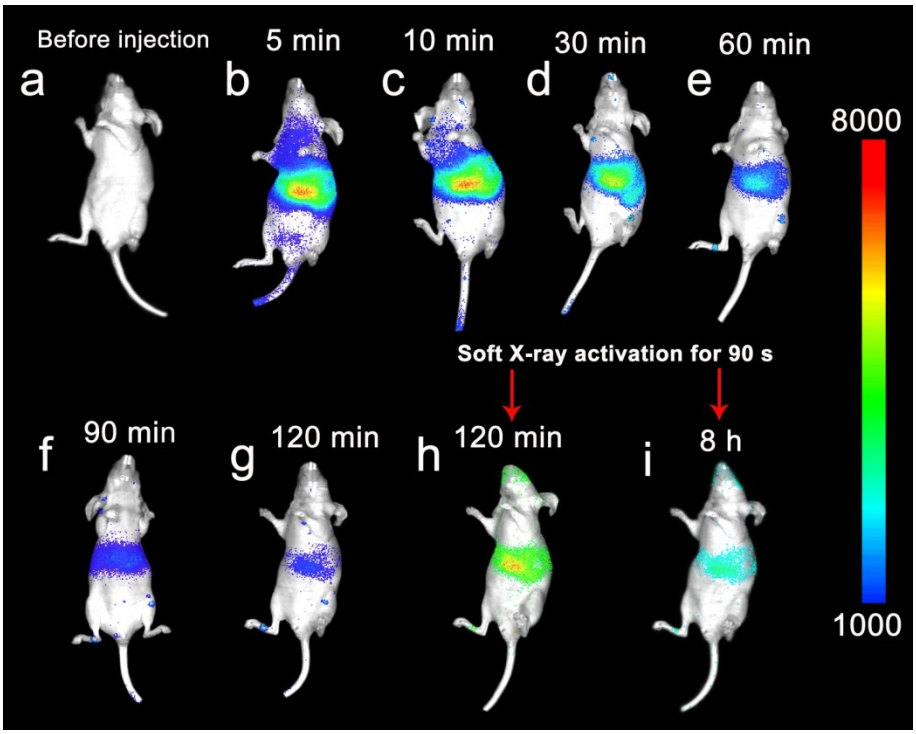


Fig. S7 (a-g) *In vivo* luminescence images of a BALB/c nude mouse after intravenous injection of of PEG-$SrAl_2O_4$:Eu^{2+} PLNPs (200 µL, 2 mg mL^{-1}, 90 s irradiation with soft X-ray activation (40 kV) before injection) and then acquired the luminescence signal after different time interval (0-120 min). (h,i) 2 h and 8 h after intravenous injection, persistent luminescence was reactivated following a 90 s soft X-ray activation (40 kV), and immediately acquired the luminescence signal. The luminescence intensity is expressed in false colour units (1 units=1000 photons s^{-1} cm^2 sr).

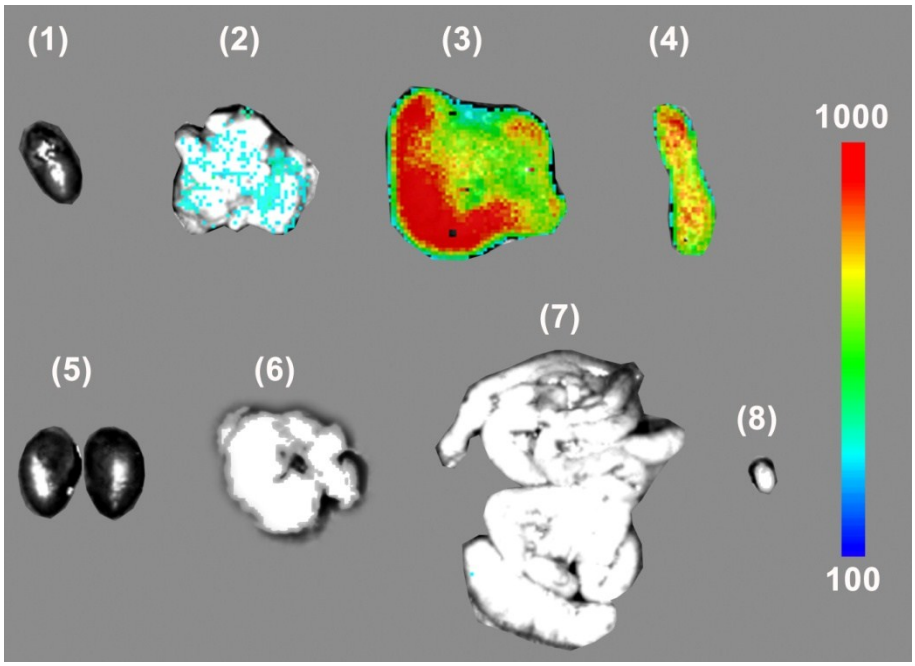


Fig. S8 Representative *ex vivo* luminescence images of isolated organs from a normal mouse at 24 h post-intravenous injection of PEG-$SrAl_2O_4$:Eu^{2+} PLNPs: (1) heart, (2) lung, (3) liver, (4) spleen, (5) kidney, (6) stomach, (7) intestine, (8) bladder. The luminescence intensity is expressed in false colour units (1 units=1000 photons s^{-1} cm^2 sr).

Supplementary References

1 P. P. Dendy and B. Heaton, *Physics for Diagnostic Radiology, Third Edition*, Taylor and France, New York, **1999**, p 22-23.