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A short-wavelength infrared emitting multimodal probe for non-invasive visualization of phagocyte cell migration in living mice

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Experimental section

Materials

Lead (II) chloride, oleylamine, and oleic acid were purchased from Wako Pure Chemical Industries. Hexamethyldisilathiane and tributylphosphine (TBP) were purchased from TCI Chemicals. Mercaptoundecanoic acid (MUA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and bovine serum albumin (BSA) was purchased from Sigma-Aldrich. Cadmium 2,4-pentanedionate (98%) was purchased from Alfa Aesar. Selenium (powder, 99.999%) and Tellurium (shot, 1-2 mm, 99.99%) were purchased from Sigma-Aldrich. Dimethylcadmium (10 wt% in hexane) was purchased from Strem Chemicals. Tetraethylorthosilicate (TEOS), Tergitol (NP-9), Fe_3O_4 magnetic particles (5 mg/mL in toluene), and rhodamine 6G (Rh6G) were purchased from Sigma-Aldrich. An inhibitor of cell migration, PTX was purchased from Sigma-Aldrich. A murine phagocytic cell marker, F4/80 antibody was purchased from Biolegend. Other chemicals used were analytical grade reagents.

Synthesis of QDs

Visible-emitting CdSe/ZnS QDs (520 nm emission) and NIR-emitting CdSeTe/CdS QDs (720 nm emission) were synthesized as described previously.[1,2]

PbS/CdS QDs were synthesized as follows.[3] Lead (II) chloride (278 mg) was dissolved in a mixture of 5 mL of oleylamine and 1 mL of oleic acid in a three-necked flask at room temperature. The solution was heated to 100-150 ^oC under an argon atmosphere. To this solution, 0.5 mL of a sulfur precursor solution (0.5 mL of hexamethyldisilathiane and 9.5 mL of TBP) was dropwisely added under vigorous

stirring. The formation of PbS QDs was monitored by measuring their emission spectra, which shifted to longer wavelengths with increasing the amount of the sulfur precursor. The reaction temperature was set to 100, 125, and 150 ^{o}C for preparing 1000, 1200 and 1400 nm emitting PbS QDs, respectively. When the desired emission peak of the PbS QDs was obtained, the solution was quickly cooled to 60 ^{o}C. Then, methanol was added to precipitate the PbS QDs. The PbS QD precipitates were dissolved in a mixture of 2 mL of oleylamine and 20 mL of toluene at room temperature. The solution was heated to 90 ^{o}C, and 0.25-0.5 mL of a Cd-S precursor solution (2.5 mL of dimethylcadmium solution + 0.5 mL of hexamethyldisilathiane + 7.5 mL TBP) was dropwisely added. By measuring the fluorescence spectra of the solution, the shell formation of CdS was monitored. When the desired emission peak of the PbS/CdS QDs was obtained, the solution was cooled to 50 ^{o}C. Then, ethanol was added to precipitate the PbS/CdS QDs, and the QD precipitations were dissolved in 20 mL of chloroform. By controlling the amounts of a Cd-S precursor solution during the shell formation, PbS/CdS QDs with the emission peaks of 1100, 1300 and 1500 nm were obtained from the PbS QDs with the emission peaks of 1000, 1200 and 1400 nm, respectively.

Water-solubilization of QDs

A chloroform solution (1m L) of the PbS/CdS QDs prepared by the above procedure was put in a rotary evaporator to remove the chloroform, and tetrahydrofuran was added to the precipitates of PbS/CdS QDs under vortex. To 1 mL of the tetrahydrofuran solution of PbS/CdS QDs (1 μM), 1 mL of a tetrahydrofuran solution of MUA (50 mg/mL) was added under stirring. To this solution, 0.5 mL of an aqueous solution (100 mg/mL potassium *t*-butoxide) was added to precipitate MUA-coated PbS/CdS QDs. The MUA-coated PbS/CdS QD precipitates were separated by centrifugation and dissolved in water. An aqueous solution of MUA-coated PbS/CdS QDs was passed through a 0.22 μm membrane filter and centrifuged at 15,000 g for 5 min to remove aggregated PbS/CdS QDs. Water-solubilization of CdSe/ZnS QDs and CdSeTe/CdS QDs were performed by the similar method.

Characterization of QDs

Fluorescence spectra of PbS/CdS QDs were measured by a NanoLog spectrometer with an InGaAs photodiode array (Symphony; HORIBA) at an excitation of 488 nm. Absorption spectra of QDs were measured with a V-670 spectrophotometer (JASCO Corporation). Fluorescence quantum yields of PbS/CdS QDs with emission peaks of 1100, 1300 and 1500 nm were estimated relatively using standard PbS QDs, which had an emission peak of 1000 nm. The quantum yield (QY) of the standard PbS QDs was

determined to be 0.6 by using an absolute quantum yield measurement system (C10027; Hamamatsu photonics). By comparing the fluorescence intensities of the standard PbS QDs and PbS/CdS QDs with OD = 0.1 at 488 nm, QYs of the PbS/CdS QDs were calculated. Molar extinction coefficients (ε) of PbS/CdS QDs were determined with the aid of fluorescence correlation spectroscopy (FCS) as follows. The surface of the PbS/CdS QDs was coated with FITC-labeled polyethyleneimine (MW 25000), and the concentration of the QDs in water was measured by FCS using a Rh6G standard solution (10^{-8} M). The molar extinction coefficient, ε, for PbS/CdS QDs in water was determined from the Lambert-Beer relationship as 6.3×10^5, 16.6×10^5 and 22.4×10^5 M^{-1} cm^{-1} for PbS/CdS with emission peaks of 1100, 1300, and 1500 nm, respectively, at an absorption wavelength of 488 nm. Hydrodynamic sizes of QDs were estimated from dynamic light scattering (DLS) using the Zetasizer Nano-ZS (Malvern) and a 633 He/Ne laser.

Synthesis of multimodal probe

The mixture (3 mL of NP-9, 200 μL of Fe_3O_4 solution and 640 μL of TEOS) was diluted with 15 mL of cyclohexane. After 30 min, 100 μL of 10% ammonia was added. The reaction proceeded by stirring for 24 hours. This reaction was performed two times. Then, 50 μL of a Rh6G chloroform solution (10 mM), 200 μL of a PbS/CdS QD chloroform solution (5 μM), and 640 μL of TEOS were added, and the reaction of the silica formation was continued for 24 hours. This reaction was performed two times. Then, 30 mL of methanol was added to the solution of silica-coated multimodal probe, and the resulting precipitates were separated by centrifugation. The precipitates were then washed by acetone and ethanol. Finally the precipitates were dispersed to a BSA solution (10 mg/mL in PBS, pH 7.4). Hydrodynamic size of the multimodal probe was estimated from DLS using the Zetasizer Nano-ZS (Malvern) with a 633 He/Ne laser.

Fluorescence imaging system

The imaging system was based on a macro zoom system with 0.63× to 6.3× magnification (MVX; Olympus). The optical system was optimized in the VIS, NIR, and SWIR region with objective and tube lenses for *in vivo* fluorescence imaging. GFP and Cy5.5 filter sets (Semrock) were used for the VIS and NIR fluorescence imaging, respectively. A filter set consisting of an excitation filter for a 785 nm laser, a dichroic mirror to reflect a 785 nm laser and transmit light over 800 nm was used for SWIR fluorescence imaging. The emission filters of 1100 ± 25 nm, 1300 ± 25 nm, and 1500 ± 25 nm placed after the filter set were used for the imaging. A Xe lamp was used as an excitation light source at 482 nm for VIS fluorescence imaging. The laser diodes with

670 nm and 785 nm emission (BWF1 series; B&W TEK) were used as excitation light sources for the NIR and SWIR fluorescence imaging, respectively. Maximum excitation powers on the sample stage were 5.0 mW/cm^2 at 482 nm excitation, 25.1 mW/cm^2 at 670 nm excitation, and 25.5 mW/cm^2 at 785 nm excitation at 0.63× magnification. A Si EM camera (iXon3, Andor) was used for VIS and NIR fluorescence imaging, and an InGaAs CMOS camera (C10633-34; Hamamatsu photonics) for SWIR fluorescence imaging.

Autofluorescence measurements

The auto-fluorescence of HOS:HR-1 (Hoshino Laboratory animals) mouse bodies was measured using a NanoLog spectrometer (HORIBA) equipped with a photomultiplier (R2658P; Hamamatsu photonics) and an InGaAs photodiode array (Symphony; HORIBA). Mice were placed on a rotatable sample chamber such that the excitation light could be introduced into the mouse body from the dorsal or ventral sides. The sample angle was regulated such that the diffuse reflection light from the mouse body did not directly enter the detection area. Long pass-filters of wavelengths 510, 710 and 810 nm (Asahi Spectra) were used at excitation wavelengths of 482, 670 and 785 nm, respectively. To normalize the autofluorescence, the excitation power was monitored during the measurements. Absorption spectrum of a mouse skin was measured using a JASCO V-670 spectrophotometer with an integrating sphere (JASCO).

In vivo fluorescence imaging

Mice were anesthetized on a microscope stage. The brightness of solutions containing BSA-coated QDs (520, 720, 1100, 1300 and 1500 nm emission) in PBS (pH = 7.4) were normalized by setting the exposure time and EM gain of the camera and the QD concentration at several μM to acquire the same average photon number per area. For lymph imaging, a 150 μL mixture of BSA-coated QDs solutions was injected into the mouse footpad at 100 μL /min. Using the above normalization setting, mouse bodies were observed at 0.63× magnification. For the non-invasive imaging of phagocyte cell migration in a mouse lymph system, a 50 μL of multimodal probe (0.5 mg /mL) containing BSA-coated QDs (1 μM, 1100 nm emission) in the absence and presence of PTX (0.5 μg) was injected into the mouse footpad at 6 hours after the injection of 500 μL of a lipopolysaccharide solution (0.1 mg/mL, Sigma-Aldrich). Then, after 0, 6 and 24 hours, the fluorescence images at 1100 and1300 nm were taken for the mouse lymph system. Magnetic resonance images by pre- and post-injection of the multimodal probe were acquired with an 11.7 T vertical bore Bruker Avance II imaging system (Bruker) that used a 12-mm home-built surface RF coil. Magnetic resonance images were acquired using a fast low angle shot (FLASH) pulse sequence and the 2D acquisition

mode (TR/TE, 400/3 ms; flip angle, 30; matrix size, 256 × 192; field of view, 12.8 mm × 9.6 mm; slice thickness, 300 μm; voxel size, 50 μm × 50 μm × 300 μm; number of average, 16). T_1 and T_2 relaxometly analysis were acquired with an 11.7 T vertical bore Bruker Avance II imaging system (Bruker).

For immunohistochemistry, mice were sacrificed at 24 hours after the injection of multimodal probes. The popliteal lymph node was fixed and embedded, and 5 μm serial sections were prepared. The sections were processed and stained with a murine phagocytic cell marker, F4/80 antibody and DAPI (BioLegend).

All animal experiments were performed in compliance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals and were approves by the Osaka University Animal Care and Use Committee.

Cell viability

HeLa cells were incubated with SWIR-emitting multimodal probe for 30 min. Then, the cells were washed with PBS and resuspended in a culture media (DMEM). A 10 μL of the cell suspension was mixed with a Trypan blue solution (10 μL), and the cell viability was determined using a cell counter (Countess; Invitrogen).

References

1 Q. Ma, Y. Nakane, Y. Mori, M. Hasegawa, Y. Yoshioka, T. M. Watanabe, K. Gonda, N. Ohuchi and T. Jin, *Biomaterials*, 2012, **33**, 8486-8494.

2 M. Hasegawa, Y. Tsukasaki, T. Ohyanagi and T. Jin, *Chem. Commun.*, 2013, **49**, 228-230.

3 Y. Tsukasaki, M. Morimatsu, G. Nishimura, T. Sakata, H. Yasuda, A. Komatsuzaki, T. M. Watanabe and T. Jin, *RSC Adv.,* 2014, **4**, 41164-41171.

Additional Figures (Figure S1- S8)

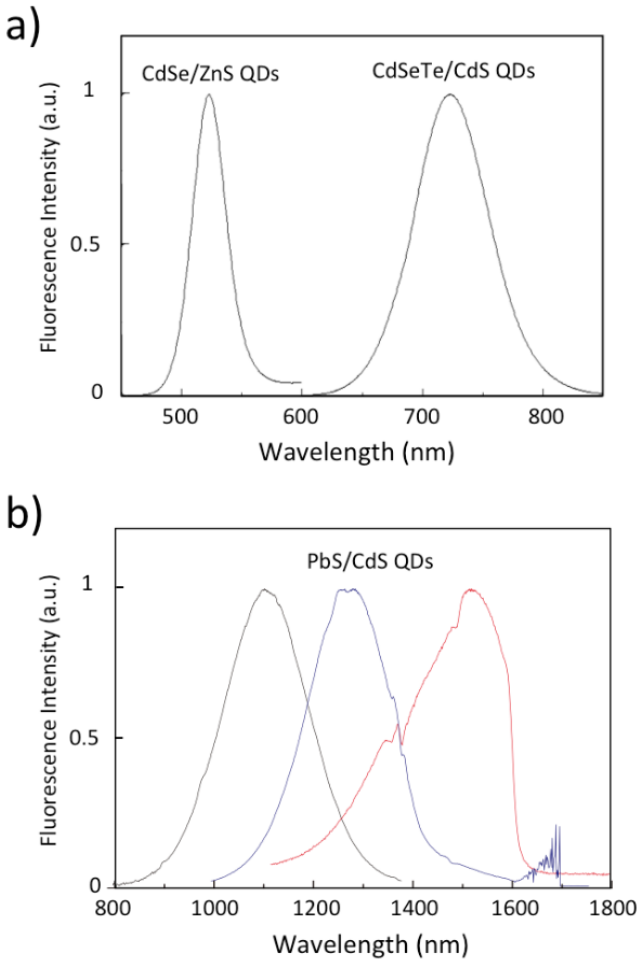

Fig. S1 Fluorescence spectra of MUA-coated QDs in water. (a) CdSe/ZnS QDs (520 nm) and CdSeTe/CdS QDs (720 nm). (b) PbS/CdS QDs (1100, 1300, and 1500 nm).

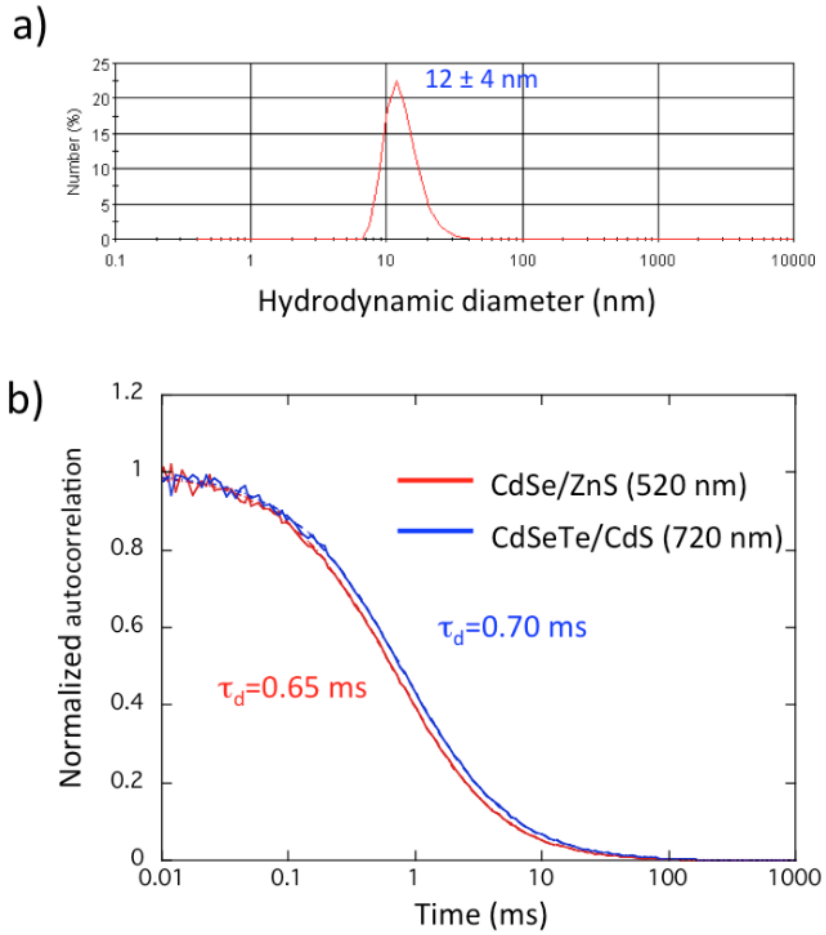

Fig. S2 (a) Hydrodynamic diameters of BSA-coated CdSe/ZnS QDs (520 nm) in water, determined by DLS. For the BSA-coated CdSeTe/CdS QDs (720 nm), their hydrodynamic diameters could not determined by DLS, due to their strong fluorescence at 633 nm, which wavelength was used for scattering measurements. (b) Fluorescence autocorrelation curves for BAS-coated CdZe/ZnS QDs (520 nm) and CdSeTe/CdS QDs (720 nm) in water. The fluorescence autocorrelation curves of BAS-coated QDs are fitted by using a single-component diffusion model, indicating monodisperse particles for the BAS-coated QDs in water.

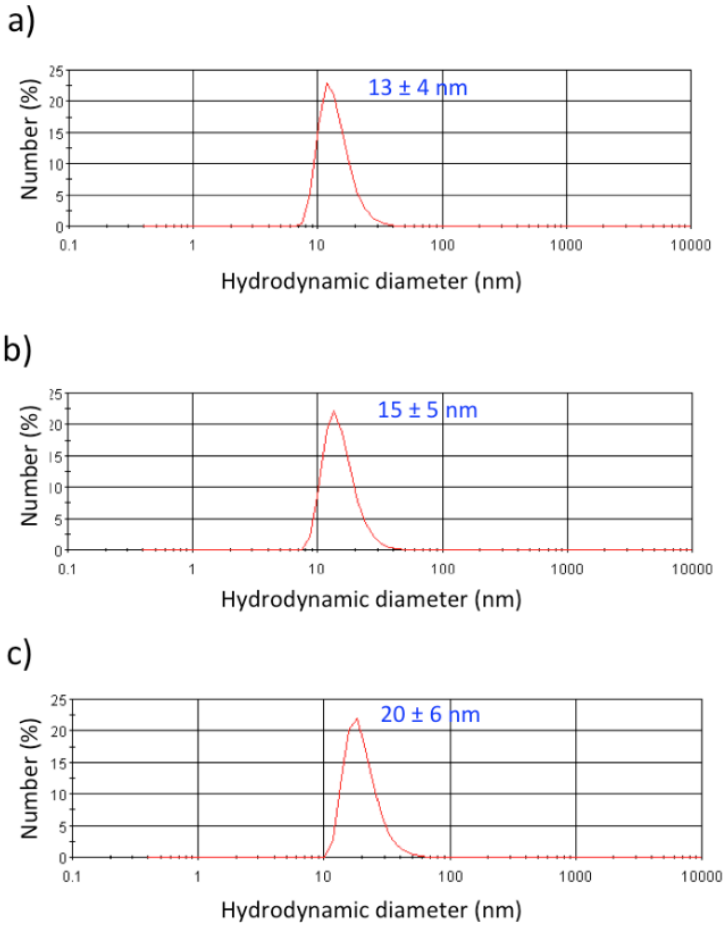

Fig. S3 Hydrodynamic diameters of BSA-coated PbS/CdS QDs with the emission peak of 1100 nm (a), 1300 m (b) and 1500 nm (c) in water, determined by DLS.

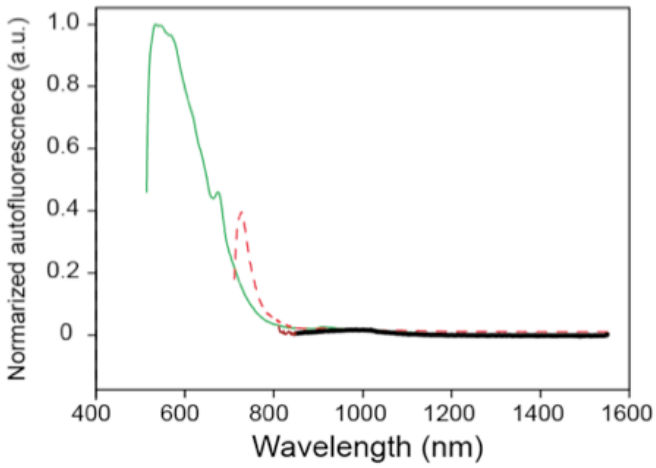

Fig. S4 Autofluorescence spectra of the dorsal side of a mouse body. The green, red, and black lines show the autofluorescence at the excitation of 482, 670 and 785 nm, respectively.

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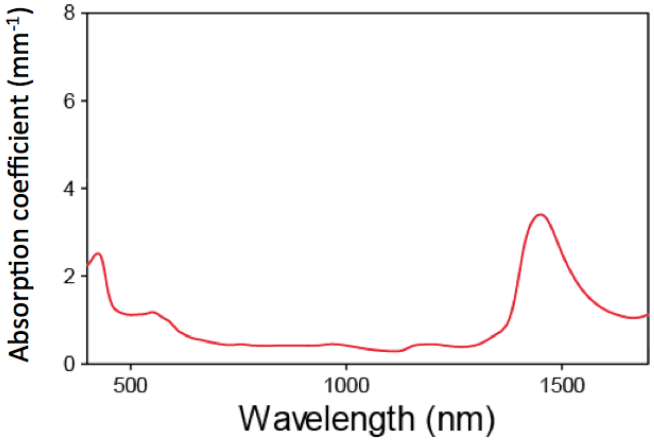

Fig. S5 Absorption spectrum of a mouse skin (120 μm thickness) measured by using an integrating sphere.

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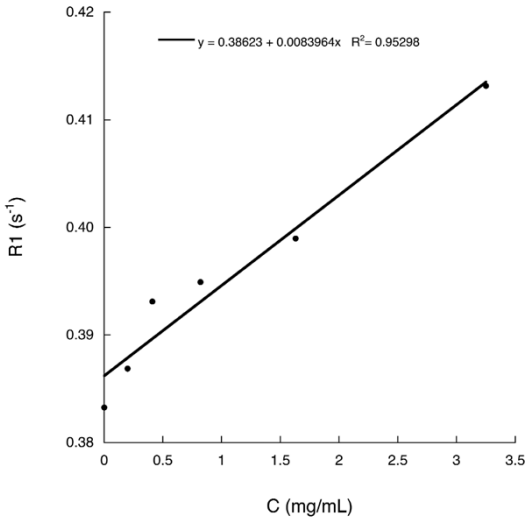

Fig. S6 T_1 relaxation rates (R_1, s^{-1}) as a function of the concentration (C) of a SWIR-emitting multimodal probe in water.

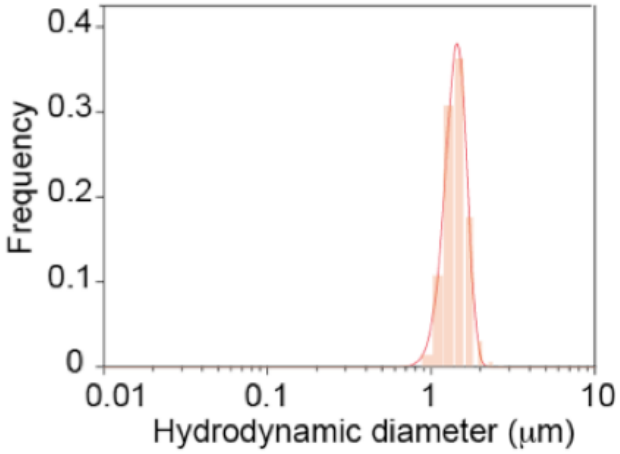

Fig. S7 Histogram of the hydrodynamic diameter of a SWIR-emitting multimodal probe in aqueous solution (10 mg/mL BSA in PBS, pH=7.4), measured by DLS.

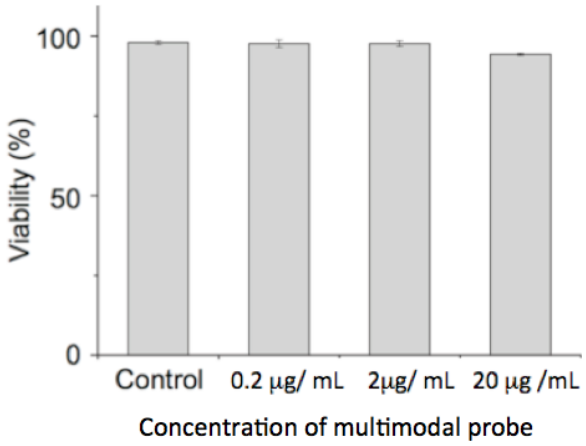

Fig. S8 Viability of HeLa cell for a SWIR-emitting multimodal probe.

Movie information

Movies S1. Timelapse lymph node imaging with BSA-conjugated QDs (1100 nm emission). Time counter is min:sec.