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A Transistor-like pH Nanoprobe for Tumour Detection and Image-guided Surgery

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Additional Materials and Methods

Characterization of monomer and polymer of PINS. Syntheses of 2-(ethylpropylamino)ethyl methacrylate (EPA-MA) and poly(ethylene glycol)-*b*-poly(ethylpropylaminoethyl methacrylate) copolymers (PEG-*b*-(PEPA)) were described in the method section in the main paper. Below are the chemical characterizations of the monomer and copolymer:

2-(Ethylpropylamino) ethyl methacrylate (EPA-MA): ^1H NMR (TMS, CDCl_3 , ppm): 6.10 (s, 1H, $\text{CHH}=\text{C}(\text{CH}_3)-$), 5.54 (s, 1H, $\text{CHH}=\text{C}(\text{CH}_3)-$), 4.20 (t, 2H, $-\text{OCH}_2\text{CH}_2\text{N}-$), 2.75 (t, 2H, $-\text{OCH}_2\text{CH}_2\text{N}-$), 2.58 (q, 2H, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$), 2.44 (m, 2H, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$), 1.94 (s, 3H, $\text{CH}_2=\text{C}(\text{CH}_3)-$), 1.45 (m, 2H, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$), 1.02 (t, 3H, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$), 0.87 (t, 3H, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$). ^{13}C NMR (CDCl_3 , ppm): 167.42, 136.36, 125.35, 63.20, 56.31, 51.51, 48.32, 20.54, 18.33, 12.09, 11.82. $[\text{M}+\text{H}]^+$: 200.2 (calculated 200.3).

Poly(ethylene glycol)-*b*-poly(ethylpropylaminoethyl methacrylate) (PEO-*b*-P(EPA)₁₀₀): ^1H NMR (TMS, CDCl_3 , ppm): 3.99 (b, 204H, $-\text{COOCH}_2-$), 3.83-3.45 (m, 450H, $-\text{CH}_2\text{CH}_2\text{O}-$), 3.38 (s, 3H, $\text{CH}_3\text{O}-$), 2.68 (b, 204H, $-\text{OCH}_2\text{CH}_2\text{N}$), 2.55 (b, 204H, $\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$), 2.41 (b, 204H, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$), 1.78-1.90 (m, 270H, CCH_3C & $\text{C}(\text{CH}_3)_2$), 1.45 (m, 204H, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$), 1.02 (b, 306, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$), 0.88 (b, 306H, $-\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)(\text{CH}_2\text{CH}_3)$). ^{13}C NMR (CDCl_3 , ppm): 177.73, 177.33, 176.61, 70.58, 63.26, 63.13, 56.21, 51.09, 45.05, 44.70, 38.69, 31.92, 30.33, 29.69, 29.36, 28.90, 23.72, 22.98, 22.69, 20.62, 16.53, 14.13, 12.18, 11.91.

Fluorescence activation of PINS. Fluorescence intensity of PINS in different pH buffer solutions was measured on a Hitachi fluorimeter (F-7500 model). For each PINS composition, a stock solution in MilliQ water at the concentration of 2.5 mg/mL was prepared. The stock solution was then diluted with either 80 mM PBS buffer with different pH values or 50% human serum in 80 mM PBS buffer with different pH values. The final micelle concentration was controlled at 0.05 mg/mL in PBS or 0.025 mg/mL in 50% human serum. The nanoprobe solution was excited at 780 nm and the emission spectra were collected from 800 nm to 900 nm. The emission intensity at 815 nm in PBS and 830 nm in 50% human serum was used to quantify the pH-response of the nanoprobes. Fluorescent images of PINS solution (0.05 mg/mL) in test tubes at different pH values were taken by a SPY Elite[®] imaging system.

Shelf-life study. Freshly prepared nanoprobe aqueous solution (5 mg/mL) was mixed with equal volume of 20% sucrose aqueous solution to generate 2.5 mg/mL stock solution in 10% sucrose. The stock solution was divided and sealed in several test tubes and frozen in a -20 °C freezer. Samples were thawed at designated time point to test the fluorescence activation in PBS or 50% human serum as described above.

Cell culture. The cancer cell lines used for *in vivo* tumour models include HN5, FaDu, HCC4034 human head and neck cancers, MDA-MB-231 and 4T1 breast cancers, U87 glioma, and HCT116 colorectal cancer cells.

HN5 and FaDu cell lines were obtained from Michael Story's lab; HCC4034 was established by John Minna's lab from a resected tumour of a head and neck patient of Dr. Baran Sumer; MD-MBA-231, 4T1 and HCT116 were obtained from David Boothman lab; U87 was obtained from Dawen Zhao lab. All cells lines were tested for mycoplasma contamination before use. Negative status for contamination was verified by Mycoplasma Detection Kit from Biotool. Cells were cultured in DMEM with 10% fetal bovine serum and antibiotics.

Transfection of GFP into HN5 cells. To set up a HN5 cell line with transgenic expression of GFP, a lentivirus transfection method was used. Briefly, transfection of HN5 cells was carried out with a MuLE expression vector plasmid encoding GFP together with a lentiviral packaging vector (psPAX2) and a vector encoding amphotropic envelope (MD2G) by lipofectamine (transfection reagent). At 24 and 48 h after transfection, the filtered lentivirus supernatant was recovered to infect target tumour cell line. After several passages post infection, target cell line was sorted for strong GFP-positive population in subsequent experiments.

Animal models. Animal protocols related to this study were reviewed and approved by the Institutional Animal Care and Use Committee. Female NOD-SCID mice (6-8 weeks) were purchased from UT Southwestern Medical Center Breeding Core. For orthotopic head and neck tumours, HN5, FaDu or HCC4034 cells (2×10^6 per mouse) were injected into the submental triangle area. One week after inoculation, animals with tumour size 100-200 mm³ were used for imaging studies. Subcutaneous breast tumour model was established by injecting MDA-MB-231 (2×10^6 per mouse) cells on the right flank. Peritoneal metastasis was established by intraperitoneal injection of HCT-116 (2×10^6 per mouse) cells followed by gentle massage on the abdomen. Orthotopic U87 glioma bearing mice were established by intracranial injection of U87 cells. Mice bearing XP296 patient-derived kidney xenograft were provided by the James Brugarolas lab. Female BalB/C mice (6-8 weeks) were purchased from UT Southwestern Medical Center Breeding Core. Orthotopic breast tumour model was established in BalB/C mice by injection of 4T1 (5×10^4 per mouse) cells into the right thoracic mammary glands.

Dose-response study. HN5-tumour-bearing mice (3 for each group) were injected with 1.0, 2.5 or 5.0 mg/kg PINS isotonic solution. The control group was injected with 0.08 mg/kg free ICG dye (equivalent to the dye content in 2.5 mg/kg PINS). At designated time point, mice were anesthetized with 2.5% isoflurane and imaged with SPY Elite[®]. Fluorescence intensity was measured by Image J. Contrast to noise ratios (CNR) were calculated by the following equation:

$$CNR = \frac{FI(Tumour) - FI(Normal Tissue)}{s.d. (Normal Tissue)}$$

$FI(Tumours)$ and $FI(Normal Tissue)$ are the fluorescence intensities of the tumour and normal tissues, respectively. The background noise was measured as the standard deviation of the normal tissue fluorescence.

***In vivo* and *ex vivo* fluorescence imaging.** Nanoprobes (2.5 mg/kg for all tumour models except 3.0 mg/kg for U87 and XP296) were administered intravenously via the tail veins of tumour-bearing mice. After 24 h, the animals were imaged by the SPY Elite® clinical camera. For *ex vivo* imaging, tumours and main organs were harvested and imaged. Fluorescence intensities of the tumours and organs were normalized to the muscle tissue of comparable size.

Cell uptake of PINS. Tumour tissue from HN5 tumour bearing mice was collected 24 h after PINS probe injection (Cy5 was used as the fluorophore in this experiment). The collected samples were frozen in OCT medium and 8 µm frozen section slides were prepared. The slides were stained with wheat germ agglutinin (WGA) and Hoechst 33258. The slides were then imaged by a DeltaVision pDV microscope. The images were merged with Image J.

Pharmacokinetics and biodistribution studies. For pharmacokinetic experiments, mice bearing HN5 tumours were injected intravenously with ³H-labelled PINS micelle solution. Blood was collected at 2 min, 30 min, 1, 3, 6, 12 and 24 h after injection. Plasma (20 µl) was isolated by centrifugation at 5,000g for 10 min. Plasma was subsequently mixed with a tissue solubilizer solution (1 mL, BTS-450; Beckman) at room temperature for 12 h followed by addition of a liquid scintillation mixture (10 mL, Ready Organic, Beckman) for 24 h. The amount of radioactive isotope was measured by a liquid scintillation counter (Beckman LS 6000 IC). After 24 h, the mice were perfused with PBS buffer (30 ml), dissected organs were weighed, homogenized and treated with scintillation mixtures. The PINS distribution in different organs/tissues was calculated as the percentage of injected dose per gram of tissue. The pharmacokinetic data were analyzed using Phoenix WinNonlin 6.0.

Safety study. Safety study was performed in immunocompetent C57BL/6 mice. Mice were injected (I.V.) with different doses of PINS (n=5 for each group). Five mice from each dose were sacrificed at day 1, 7 and 28. The control group (n=5) was injected with PBS and sacrificed after 28 days. For repeated injection study, 5 mice were injected at 50 mg/kg PINS per week for 5 weeks and were sacrificed after 6 weeks. The blood samples (~0.8-1 mL/mouse) were collected from all mice and centrifuged at 5,000 g for 10 min to separate serum. Liver function was evaluated based on the serum levels of alanine transaminase (ALT) and glutamic oxaloacetic transaminase (GOT). Kidney function was determined by blood urea nitrogen (BUN) and creatinine (CRE). The tissues of heart, liver, spleen and kidney were collected and immediately fixed in 10% formalin for histopathological examinations.

Supplementary Table 1. Characterization of PEPA_x-ICG₁ copolymers with different repeating units of PEPA segment but the same ICG content and the resulting nanoprobe properties.

copolymer	M _n (kDa) ^a	Repeating unit ^b	Particle size (nm)	pH _t	ΔpH _{10-90%}	FI _{HS6.5} ^c
PEPA ₄₀ -ICG ₁	13.5	43	21.9±1.7	6.96	0.30	32.3
PEPA ₆₀ -ICG ₁	16.8	62	24.8±0.9	6.94	0.25	37.0
PEPA ₈₀ -ICG ₁	19.7	79	25.3±0.8	6.92	0.18	45.3
PEPA ₁₀₀ -ICG ₁	25.1	102	26.0±1.1	6.92	0.15	49.3
PEPA ₁₂₀ -ICG ₁	29.1	119	27.6±1.0	6.91	0.13	51.6

^aNumber-averaged molecular weights (M_n) were determined by GPC using THF as the eluent;

^bRepeating unit was calculated based on integrations of -CH₂-O- groups on PEPA to the methylene groups on PEG using ¹H NMR; ^cDetermined as ICG fluorescence emission intensity in 50% human serum.

Supplementary Table 2. Characterization of PEPA₁₀₀-ICG_y copolymers with different ICG content but the same repeating unit of PEPA and the resulting nanoprobe properties.

copolymer	ICG conjugation number ^a	Particle size (nm)	pH _t	ΔpH _{10-90%}	FI _{HS6.5} ^b
PEPA ₁₀₀ -ICG _{0.5}	0.4	25.6±1.1	6.92	0.26	41.7
PEPA ₁₀₀ -ICG ₁	1.1	26.0±1.1	6.92	0.15	49.3
PEPA ₁₀₀ -ICG ₂	1.8	26.0±1.8	6.92	0.14	35.2

^aDetermined by a standard curve base on UV of the free ICG in methanol. ^bDetermined as ICG fluorescence emission intensity in 50% human serum.

Supplementary Table 3. Tumour fluorescence intensity (FI) and contrast over noise ratio (CNR) for each tumour bearing mouse in dose-response study.

Time	1.0 mg/kg			2.5 mg/kg			5.0 mg/kg		
	Mouse No.	Tumour FI (a.u.)	CNR	Mouse No.	Tumour FI (a.u.)	CNR	Mouse No.	Tumour FI (a.u.)	CNR
5 min	1	5.1	0.08	1	1.2	0.21	1	14.1	1.1
	2	4.6	0.12	2	3.9	1.3	2	2.6	0.70
	3	10.8	0.68	3	1.8	0.38	3	3.1	2.9
30 min	1	7.8	0.42	1	8.5	2.2	1	39.3	4.4
	2	5.3	0.06	2	6.8	1.7	2	16.0	2.6
	3	11.1	0.79	3	7.0	1.7	3	15.3	5.5
1 h	1	9.7	0.82	1	11.2	2.9	1	61.6	5.9
	2	6.4	0.18	2	12.0	2.6	2	36.3	7.0
	3	11.6	0.83	3	14.9	4.0	3	27.9	5.7
3 h	1	20.9	2.1	1	60.6	9.9	1	165	18.5
	2	15.5	1.4	2	44.9	8.9	2	117	15.1
	3	27.9	2.6	3	62.4	11.3	3	103	16.6
6 h	1	46.9	5.3	1	65.9	18.2	1	143	19.6
	2	33.5	3.1	2	42.2	16.8	2	123	18.2
	3	50.4	4.5	3	60.1	19.1	3	108	21.7
12 h	1	72.4	7.8	1	125	28.0	1	214	18.7
	2	70.9	6.7	2	83.4	28.0	2	179	20.2
	3	95.2	8.5	3	124	26.9	3	204	21.3
24 h	1	92.0	9.8	1	178	26.2	1	218	16.6
	2	59.3	5.2	2	130	27.0	2	193	19.6
	3	71.9	4.9	3	169	28.6	3	202	16.5

Supplementary Table 4. Quantitative results of ^{18}F FDG tumour uptake in mice bearing large and small HN5 tumours.

Tumour size	Mouse	Weight (g)	Tumour volume (mm ³) ^a	%ID/g	SUV	SUV _{max}
Large tumour	1	17.4	192	7.2	1.3	2.2
	2	15.3	199	8.9	1.4	2.5
	3	21.0	210	7.8	1.6	2.8
Small tumour	1	17.6	9.7	8.1	1.4	2.0
	2	20.5	11.8	7.3	1.5	2.0
	3	20.0	11.4	6.0	1.2	2.1

^aDetermined by CT imaging.

Supplementary Table 5. Sensitivity and specificity assessment for PINS-guided resection of HN5 tumours.

		Cancer (by histology)	
		Positive	Negative
PINS fluorescence	Positive	18 (TP)	0 (FP)
	Negative	0 (FN)	9 (TN)
		$Sensitivity = TP / (TP + FN)$ $= 100 \%$	$Specificity = TN / (TN + FP)$ $= 100 \%$

TP= True Positive; FP = False Positive; FN = False Negative; TN = True Negative

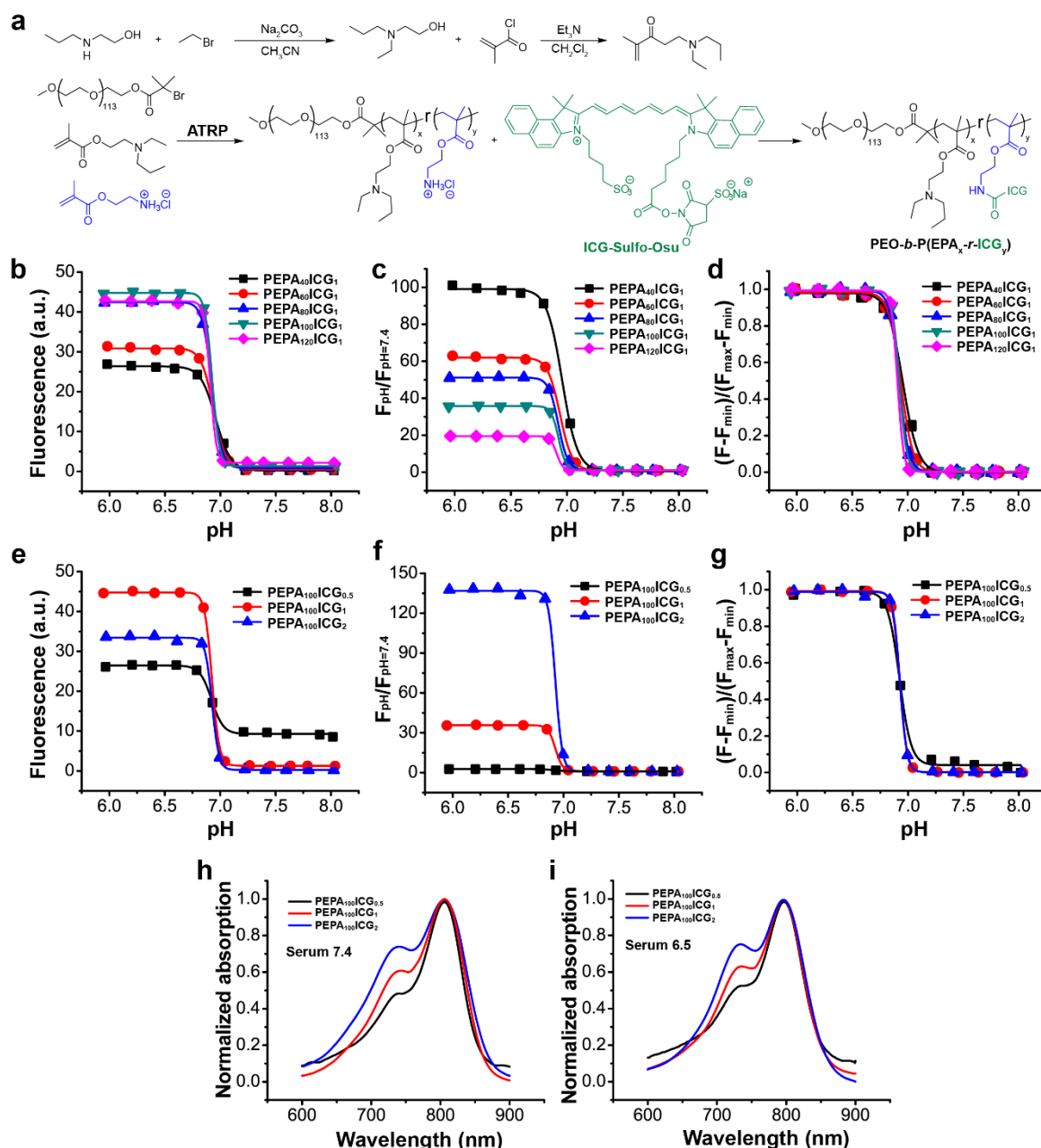
Supplementary Table 6. Log-rank *p*-values for pairwise treatment comparisons among different groups in survival surgery.

		Debulking	White light	TAGS
Head and neck surgery	Control	0.347	<0.0001	<0.0001
	Debulking	-	<0.0001	<0.0001
	White light	-	-	<0.0001
Breast surgery	Control	-	0.0501	<0.0001
	White light	-	-	0.0116

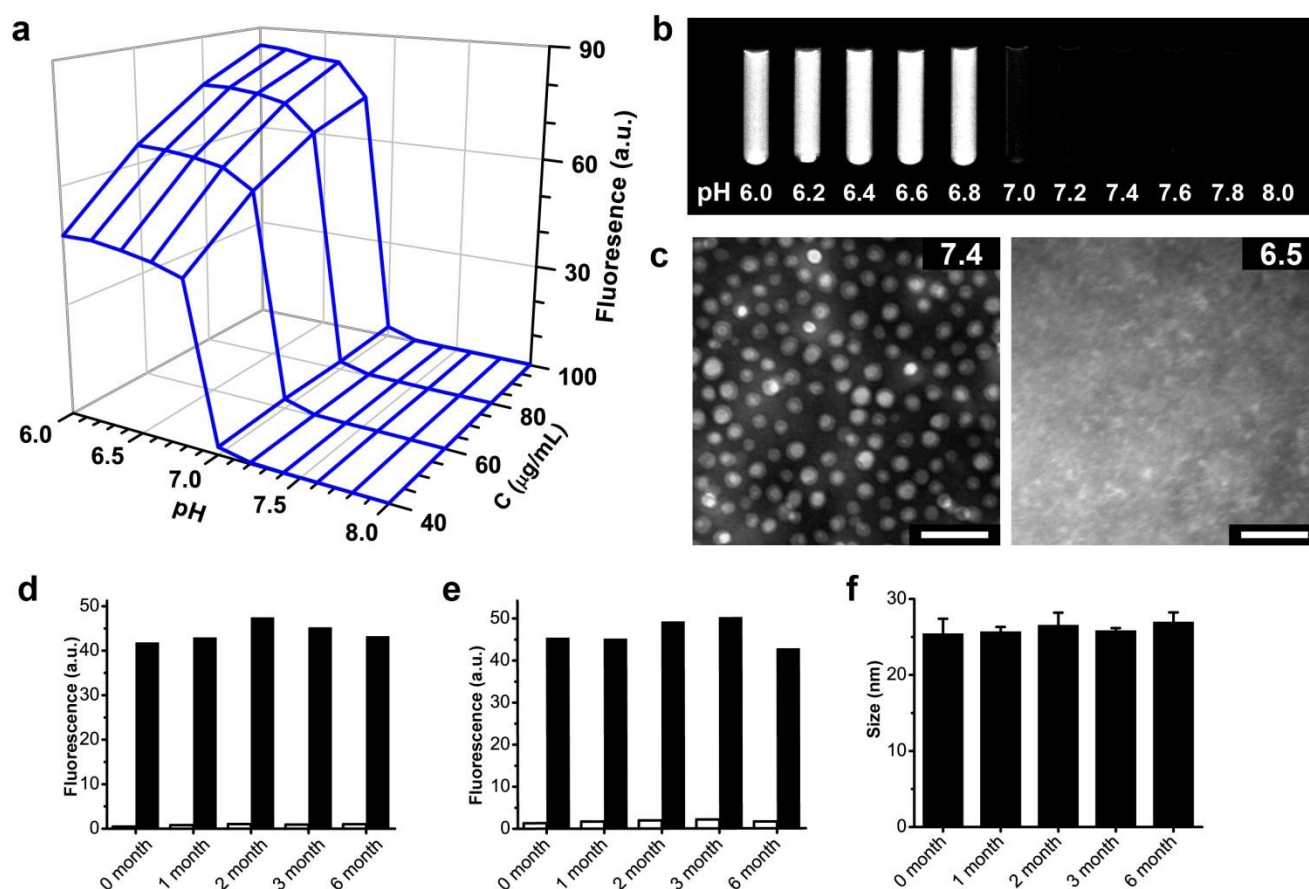
Supplementary Table 7. Tolerability and survival of C57BL/6 mice following bolus injection of PINS.

Dose (mg/kg)	7 Days mortality: n death/total n (% deaths)	Morbidity reaction type and degree ^a		
		Reduced feces	Lack of mobility (< 6 h)	Lack of appetite (< 6 h)
150	0/5 (0%)	-	recovered soon	recovered soon
200	0/10 (0%)	+	+	+
250	0/15 (0%)	++	++	++
300	4/5 (80%)	+++	+++	+++

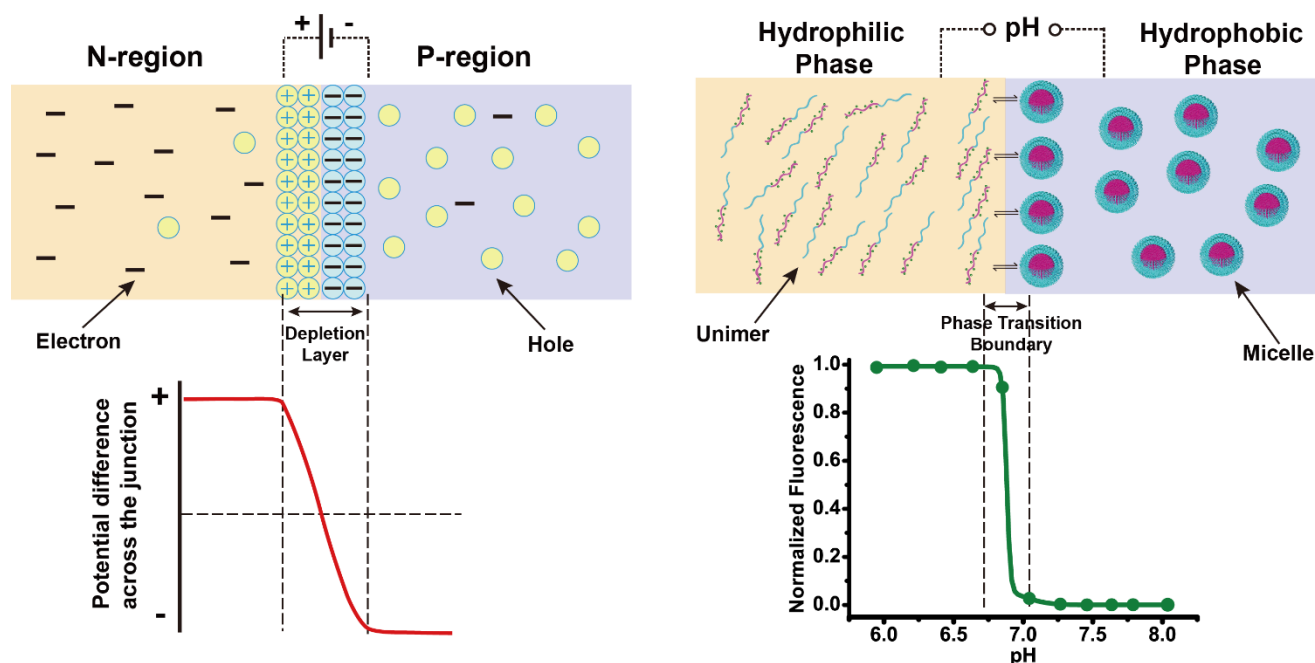
^aReaction degree was recorded as: - no reaction; + mild reaction; ++ intermediate reaction; +++ strong reaction.



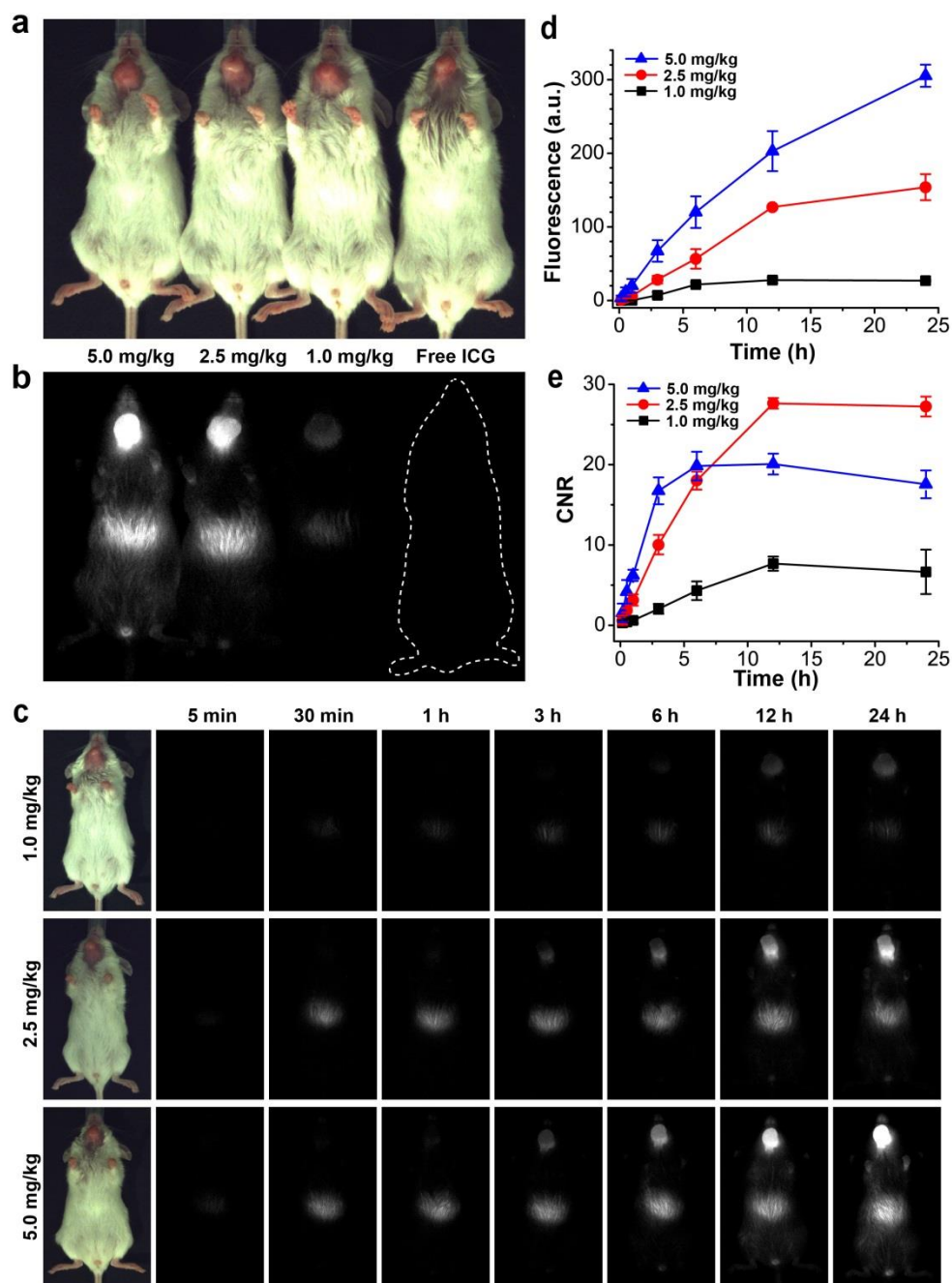
Supplementary Figure 1 | Syntheses and optimization of PINS nanoprobe. **a**, Schematic syntheses of ICG-conjugated PEG-*b*-P(EPA_x-*r*-ICG_y) block copolymers. **b-d**, Investigation of the influence of the PEPA segment length on the pH-dependent fluorescence properties: (b) fluorescence intensity, (c) fluorescence activation ratio at pH of interest over 7.4, and (d) normalized fluorescence intensity. The PEPA segment length was varied ($x = 40, 60, 80, 100, 120$) while the number of ICG per polymer chain was maintained at 1. **e-g**, Investigation of the influence of ICG conjugation number on the pH-dependent fluorescence properties: (e) fluorescence intensity, (f) fluorescence activation ratio at pH of interest over 7.4, and (g) normalized fluorescence intensity. The number of ICG per polymer chain was varied ($y = 0.5, 1, 2$) while the PEPA segment length was controlled at 100. **h, i**, UV-Vis absorption spectra with normalization to the monomer peak intensity ($\lambda = 808$ nm) of PEPA₁₀₀-ICG_y ($n = 0.5, 1, 2$) in (h) human serum at pH 7.4 and (i) human serum at pH 6.5.



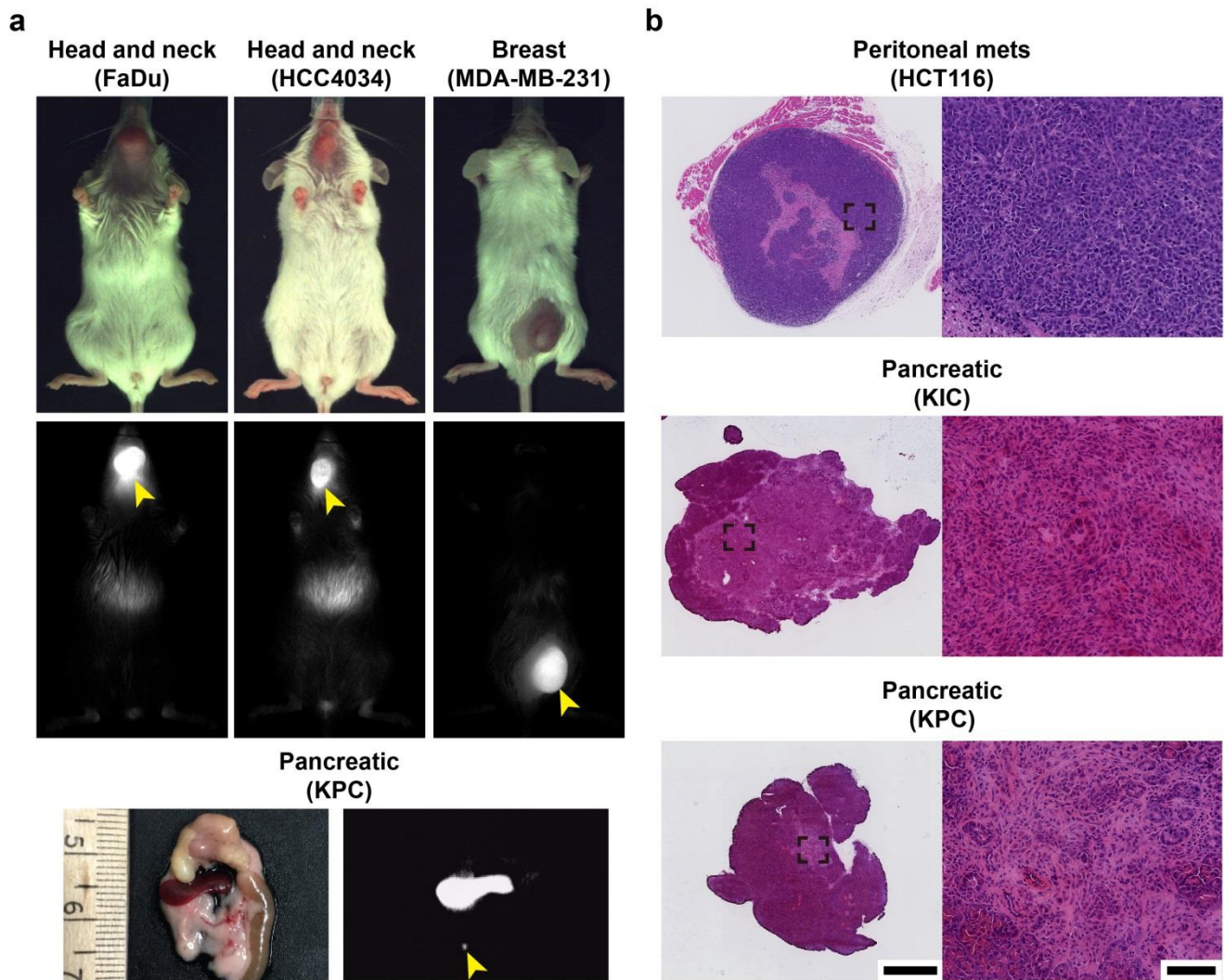
Supplementary Figure 2 | Characterization of PINS. **a**, A 3D plot of fluorescence intensity as a function of PINS concentration and pH. **b**, Near IR images of PINS solution by SPY Elite[®] surgical camera showing pH-sensitive off/on activation. **c**, Transmission electron micrographs of PINS in the micelle and unimer states at pH 7.4 and 6.5, respectively. Polymer concentration = 1 mg/mL; scale bars = 100 nm. PINS fluorescence intensity at pH 6.5 (black bars) and 7.4 (white bars) in PBS (**d**) or 50% human serum (**e**) upon storage. **f**, Number-weighted hydrodynamic radius of PINS nanoprobe upon storage (n=3). Storage condition for (**d**)–(**f**): 10% w/v sucrose solution at -20 °C. These results show PINS was stable in storage over 6 months in 10% w/v sucrose solution at -20 °C.



Supplementary Figure 3 | Analogy between an electronic transistor vs. pH nanotransistor. Both systems amplify/switch signals and arise from nanoscale phenomenon due to phase separation. Electronic transistors consist of P-doped and N-doped semiconductors that upon contact, form a depletion layer acting as a barrier for electron flow. For pH transistor nanoprobe, protonated unimer state and neutral micelle state are found across the transition pH to modulate the fluorescence signal. Threshold voltage and pH_t values serve as gating signals for electronic transistor and pH nanotransistor, respectively.

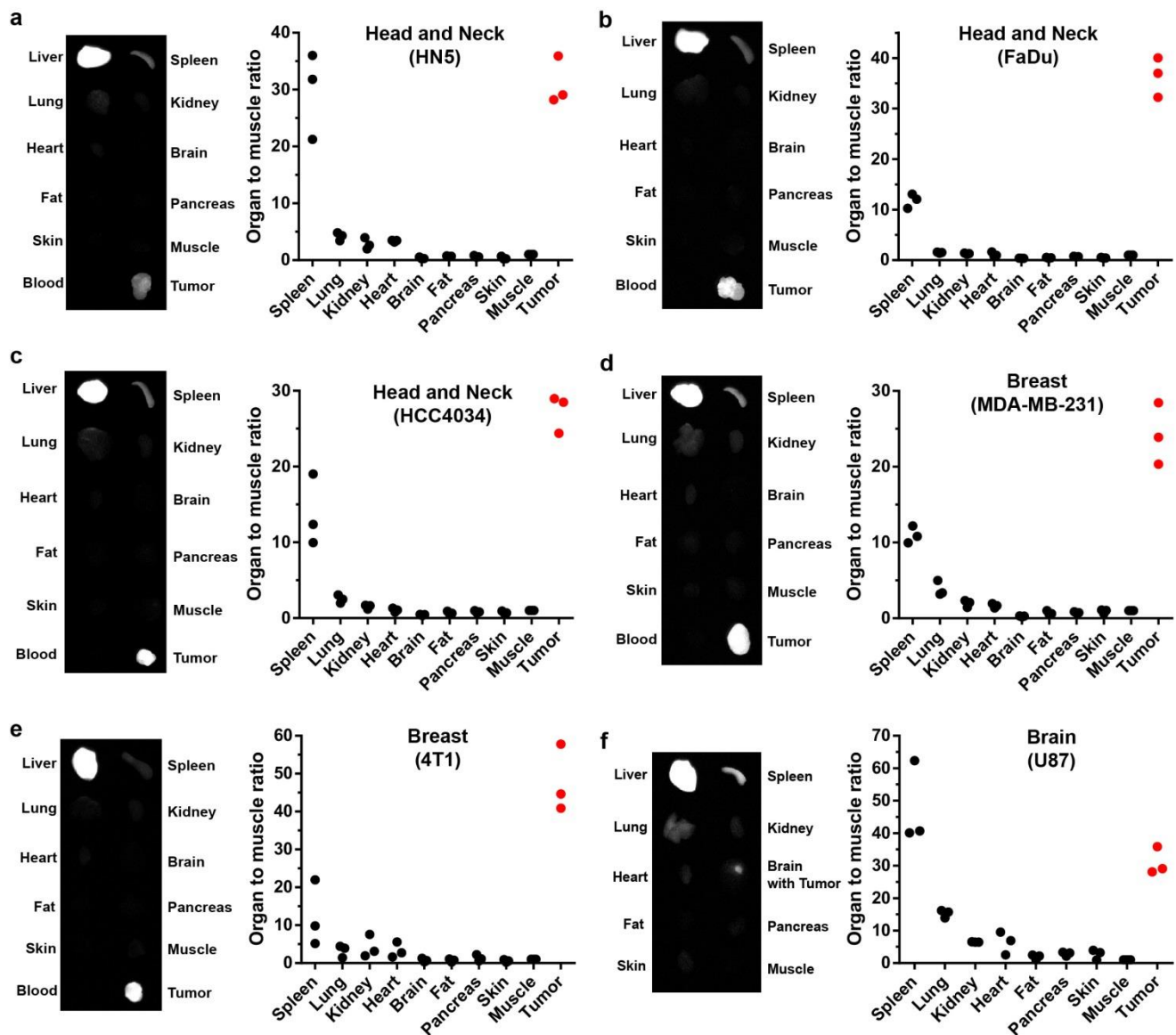


Supplementary Figure 4 | Dose-response of PINS in mice bearing human HN5 orthotopic tumours. White light (a) and near IR (b) images of mice injected with different doses of PINS (1.0, 2.5 and 5.0 mg/kg) via the tail veins. HN5 tumour intensity increased with increasing PINS dose. Free ICG control at an equivalent dye dose to 2.5 mg/kg PINS did not show observable tumour contrast. c, NIR images of representative mice injected with different doses of PINS at selected time points. Quantification of tumour fluorescence intensity (d) and tumour contrast over noise ratio (e) as a function of time after intravenous injection (n = 3). Higher PINS dose at 5.0 mg/kg led to reduced CNR value due to the higher background signal in muscle tissue. Based on results from e, we chose 2.5 mg/kg as the optimal PINS dose for tumour acidosis imaging.

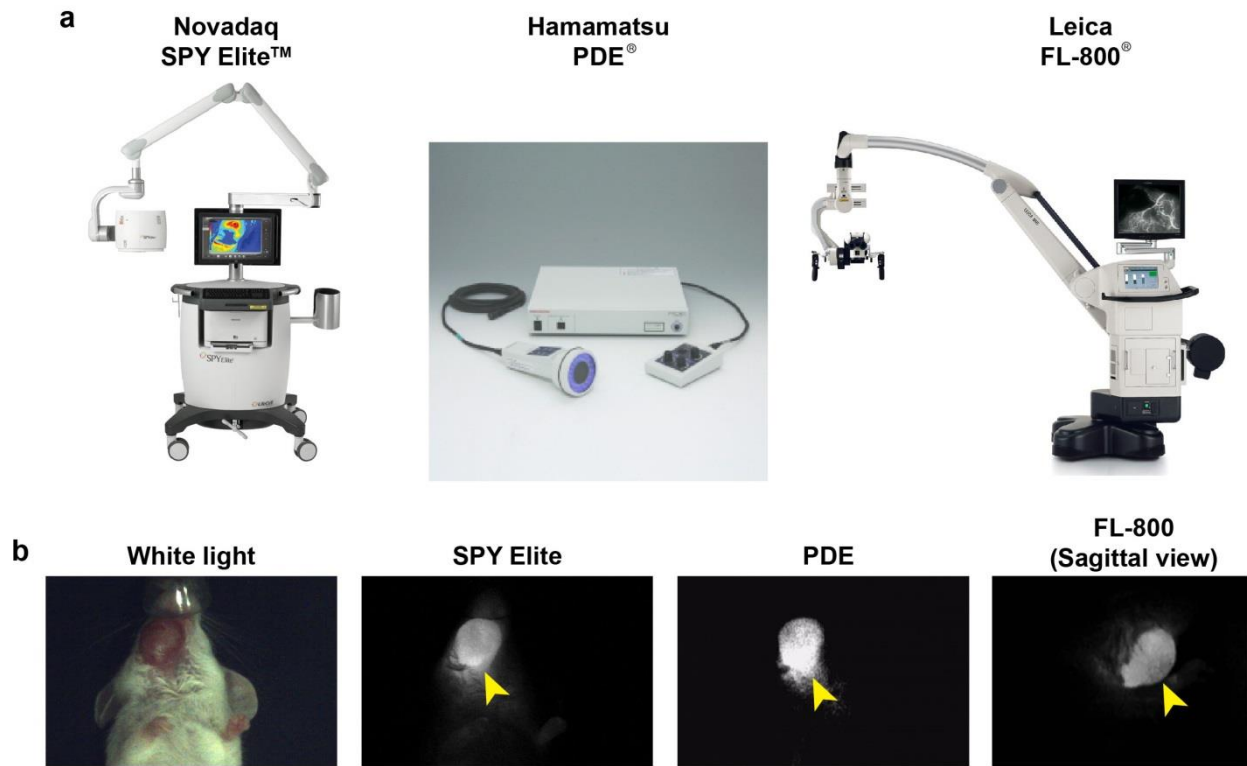


Supplementary Figure 5 | PINS imaging illuminates additional cancer models with high tumour contrasts.

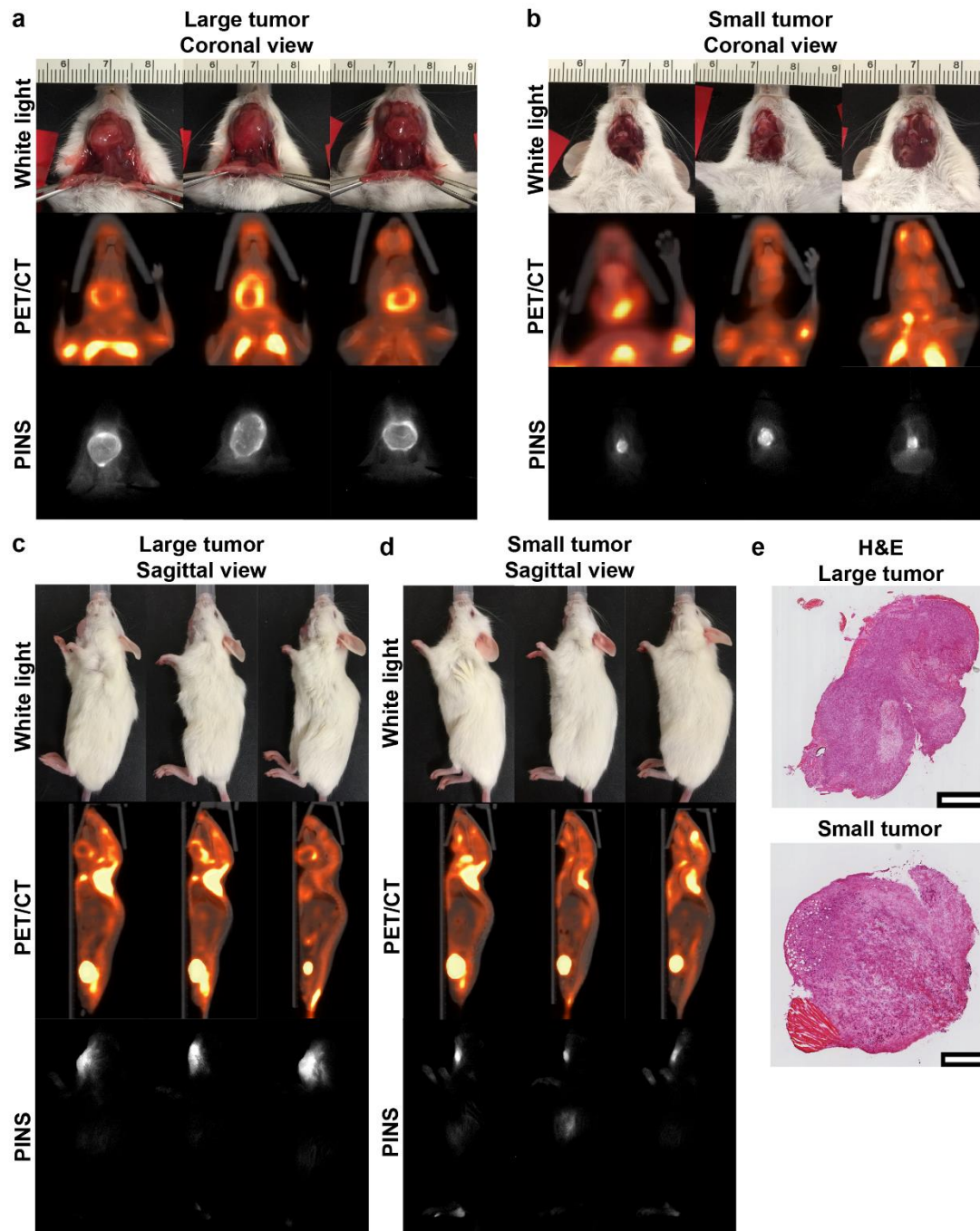
(a) PINS nanoprobes demonstrate broad tumour imaging specificity in additional tumour models (head and neck, breast and transgenic pancreatic cancers). Yellow arrow heads indicate the location of tumours. (b) Histology validation of peritoneal mets and transgenic pancreatic tumours. Scale bar = 1 mm (low magnification) or 100 μ m (high magnification).



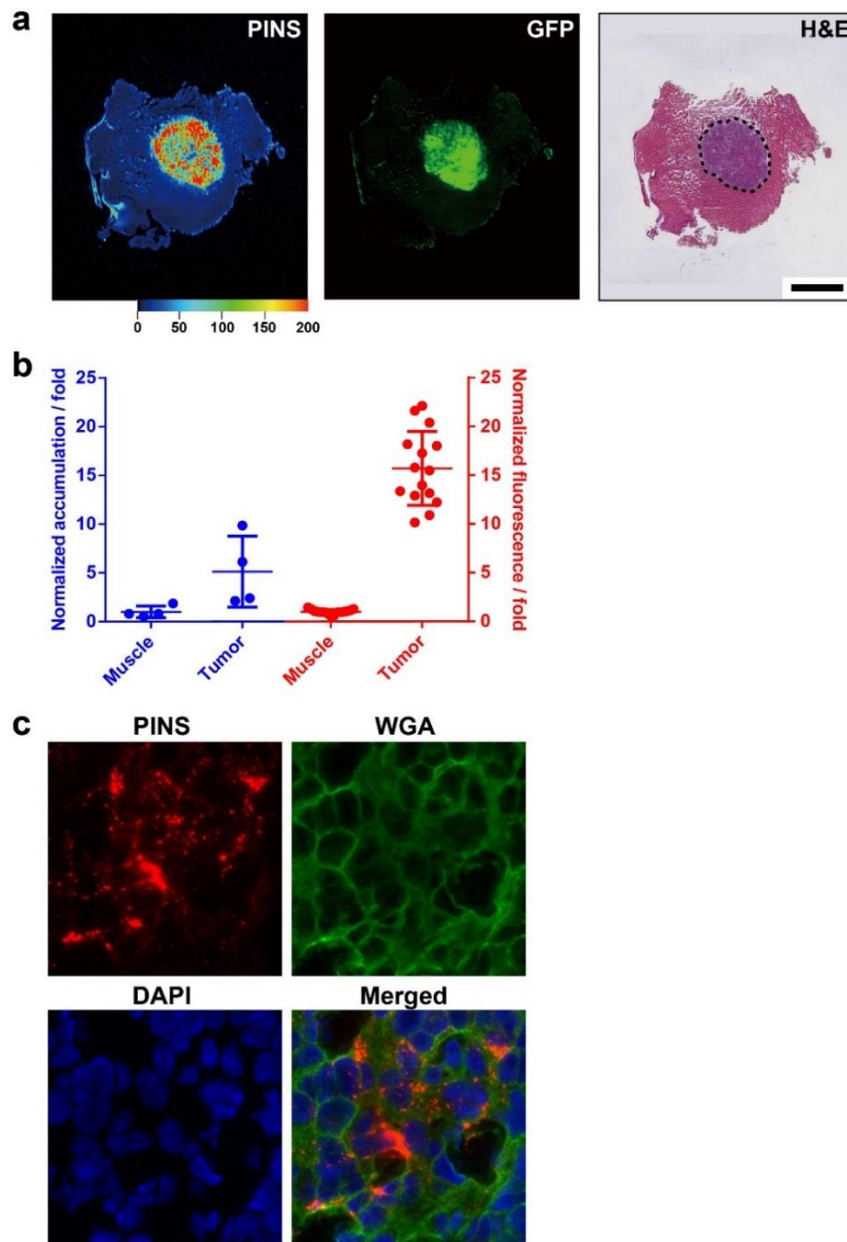
Supplementary Figure 6 | *Ex vivo* organ and tumour fluorescence imaging after PINS injection. NIR images of main organs and quantification of organ to muscle ratios of fluorescence intensity 24 h after injection of nanoprobe in mice bearing (a) HN5, (b) FaDu and (c) HCC4034 head and neck tumours, (d) MBA-MD-231 and (e) 4T1 breast tumours, and (f) U87 glioma. Data were plotted as individual data points according to the statistical guideline ($n = 3$). Livers were not calculated due to signal saturation.



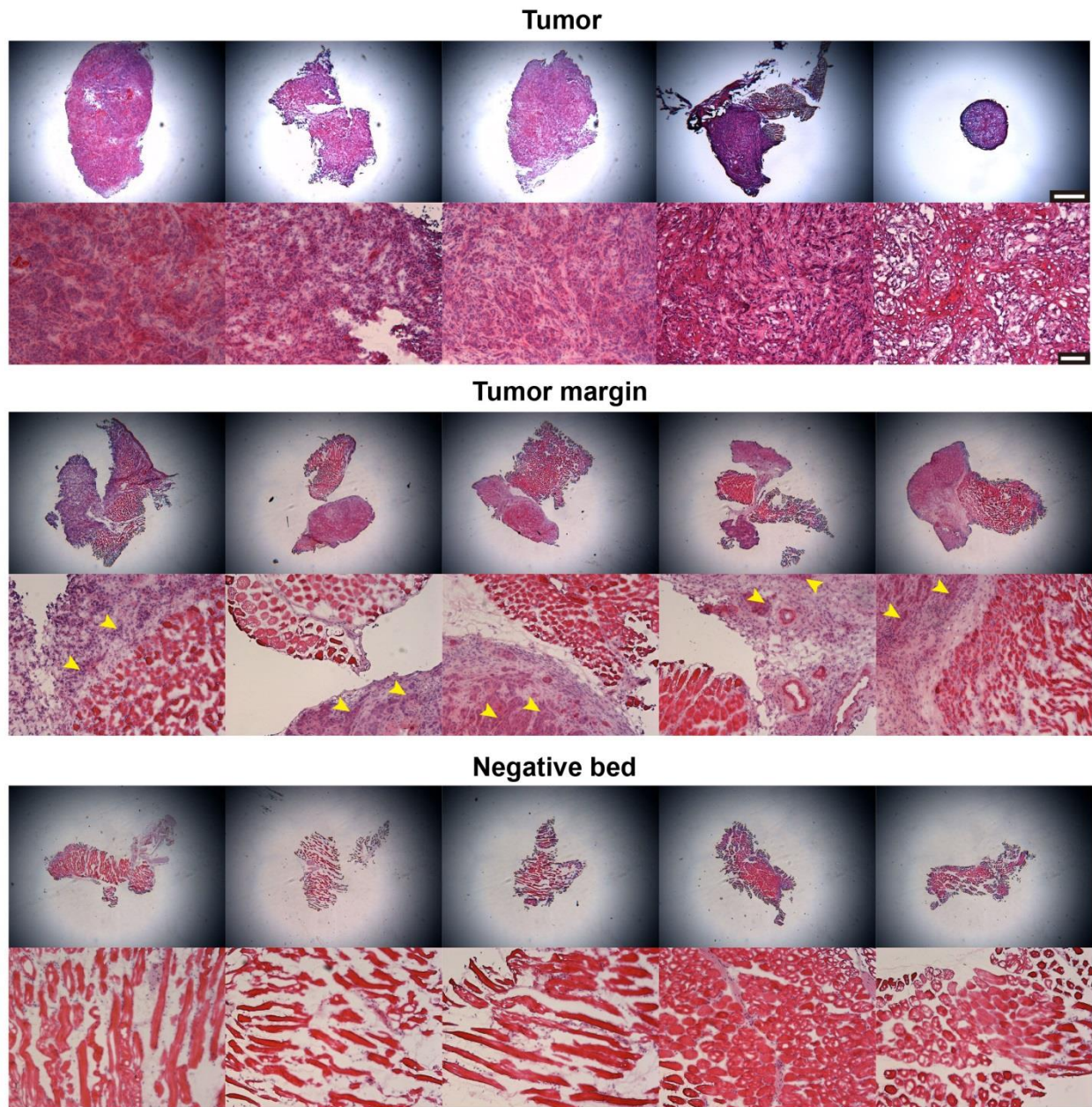
Supplementary Figure 7 | Compatibility of PINS nanoprobes with different clinical cameras. a, Clinically used ICG imaging systems: Novadaq SPY Elite®, Hamamatsu PDE and Leica FL-800 models. **b,** White light and NIR images of the same tumour bearing mouse under different clinical ICG imaging systems.



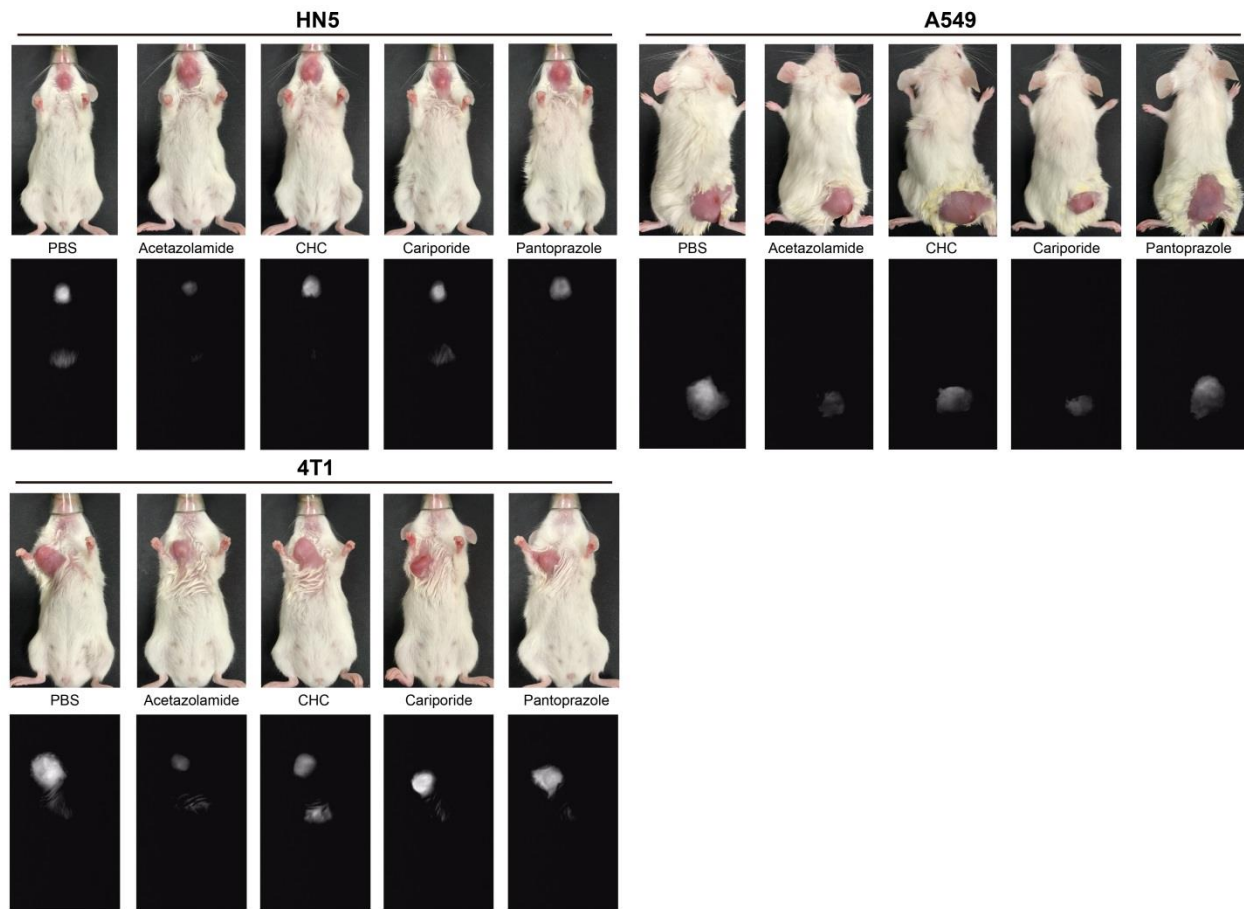
Supplementary Figure 8 | Comparison of FDG-PET with PINS imaging in mice bearing orthotopic HN5 tumours. White light, FDG-PET/CT and NIR images for the same group of mice with large tumours (200 mm³, **a**) or small tumours (10 mm³, **b**). PINS imaging allowed clear tumour margin delineation for all large and small tumours. For large tumours, FDG-PET showed higher signal on the periphery of the tumours consist with PINS activation. The asterisks indicate high FDG uptake in eye muscle from this selected PET section. Similar eye activity was also observed in other mice. Sagittal view of the same group of mice with large (**c**) and small tumours (**d**). **e**, Stitched H&E images for the large and small tumours shown in **Fig. 1**. Scale bars: 2 mm in large tumour and 500 μ m in small tumour images.



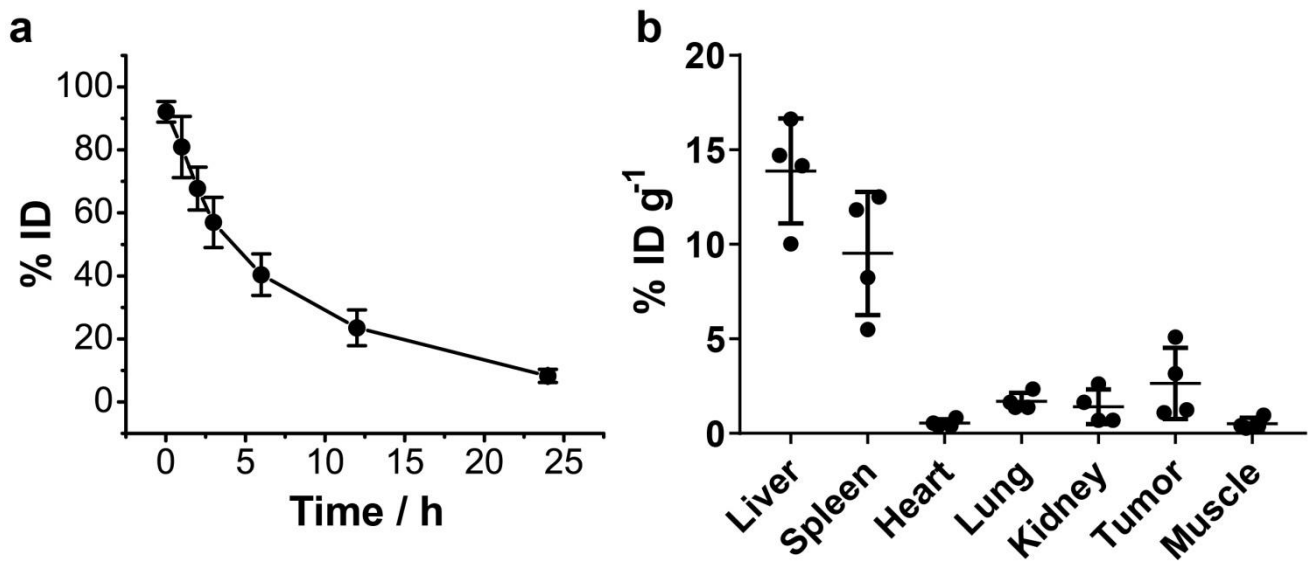
Supplementary Figure 9 | Colocalization of PINS fluorescent signal with tumour and surrounding normal tissue and signal amplification by PINS. a, Representative frozen section of HN5 tumour with surrounding tissues showed excellent matching of PINS signal with GFP-labelled HN5 cancer cells and H&E tumour histology; scale bar = 2 mm. Dashed line indicates the tumour margin. **b,** Normalized PINS accumulation in tumour and muscle tissue as measured by radioactivity from ^3H -labelled PINS (data points shown in blue, $n = 4$) and normalized fluorescence intensity measured on different frozen section slides (8 μm in thickness) from tumour and surrounding muscle tissue (data points shown in red, $n = 15$). **c,** Representative frozen section of HN5 tumour shows internalization of PINS (Cy5 as the fluorophore) by cancer cells 24h after injection. The cell membrane was stained by wheat germ agglutinin (WGA) and the nuclei by Hoechst.



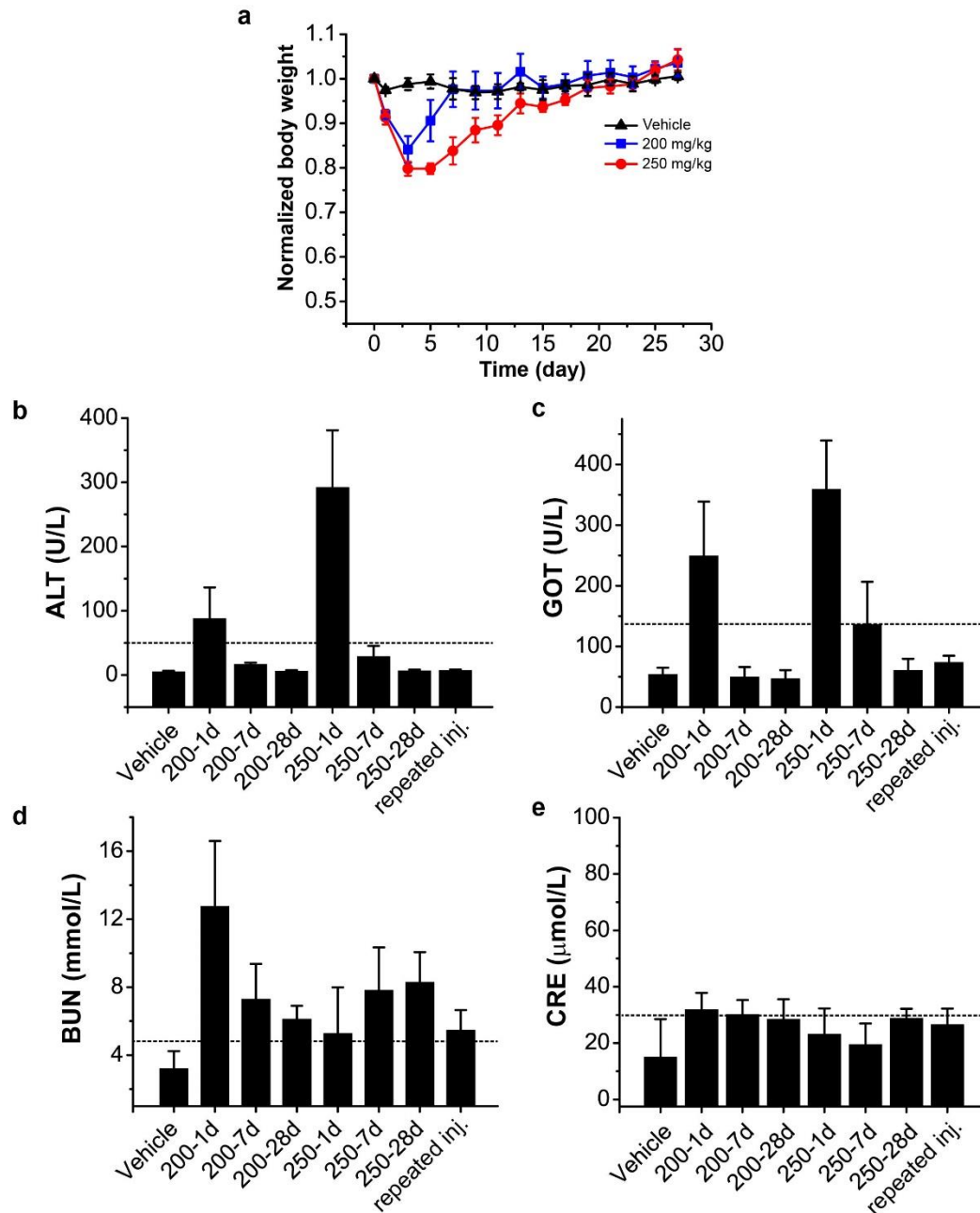
Supplementary Figure 10 | Histology validation of primary tumour, tumour margin and negative bed. Five representative H&E histology images from each type of specimens collected during the non-survival surgeries. Yellow arrow heads indicate the presence of cancer cells in the tumour margin specimens. Scale bar = 1 mm (top rows, low magnification) or 100 μ m (bottom rows, high magnification).



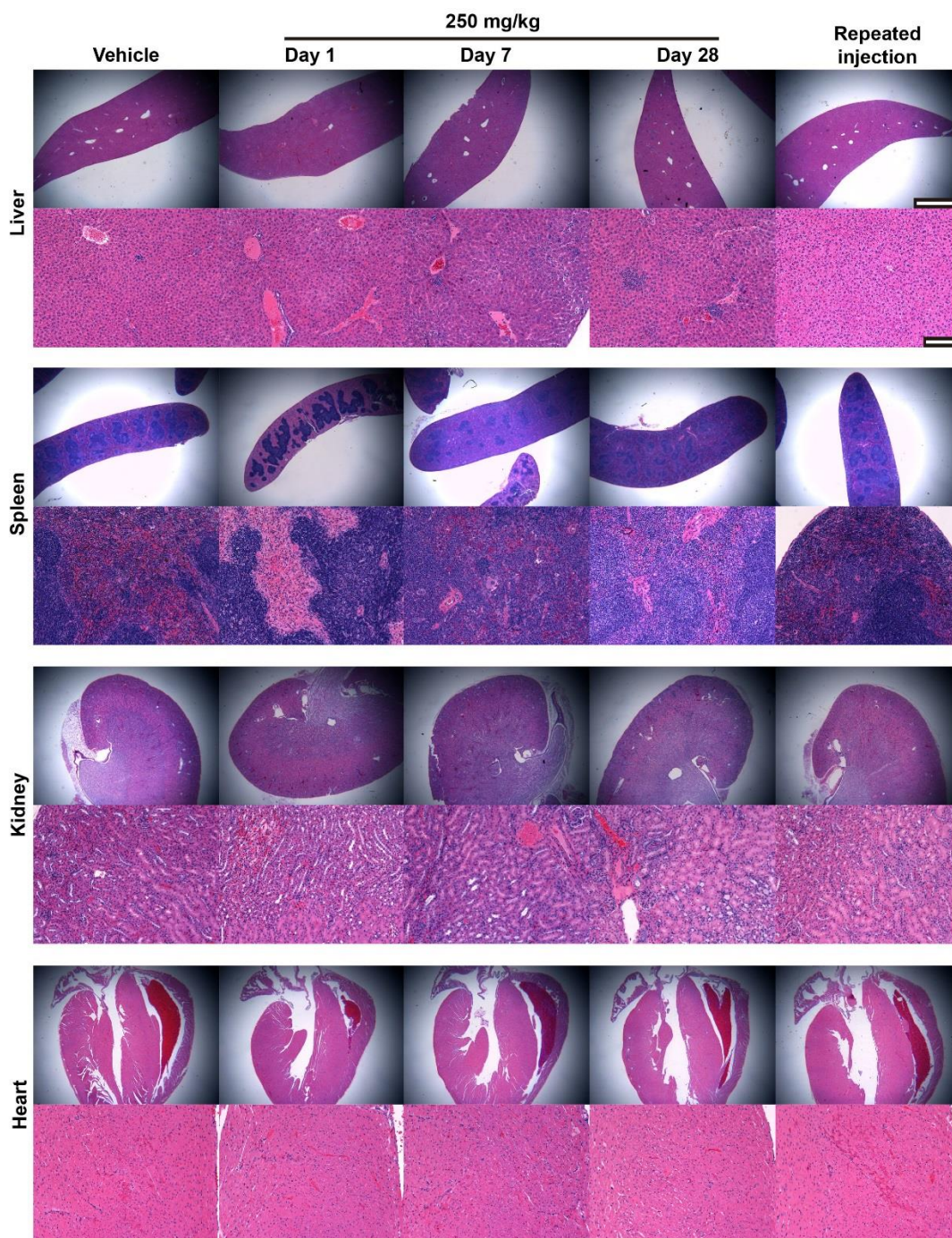
Supplementary Figure 11 | Evaluation of small molecular inhibitors targeting different tumour acidosis pathways by PINS. Representative white light and NIR images of mice bearing orthotopic HN5 head/neck tumours, orthotopic 4T1 breast tumours and subcutaneous A549 lung tumours after injection of PBS or other tumour acidosis inhibitors.



Supplementary Figure 12 | *In vivo* pharmacokinetic biodistribution studies of ³H-labeled PINS nanoprobe in HN5 tumour-bearing mice. a, Plasma concentration versus time curve of PINS nanoprobe (n = 4). **b,** Biodistribution profile in different organs (n = 4) of PINS nanoprobe 24 h after intravenous injection.



Supplementary Figure 13 | Safety assessment of intravenously administered PINS in healthy C57BL/6 mice. **a**, Normalized change of body weight of C57BL/6 immunocompetent mice after bolus injection of 200 or 250 mg/kg PINS compared to PBS control. **b-e**, Serum tests for liver (**b** and **c**) and kidney (**d** and **e**) functions of C57BL/6 immunocompetent mice after bolus injection of PINS at different doses and sacrificed after selected time points. For all groups $n = 5$; data are presented as mean $\pm$ s.d.. Abbreviations: ALT, alanine aminotransferase; GOT, glutamic oxaloacetic transaminase; BUN, blood urea nitrogen; CRE, creatinine; dotted lines indicate typical wild-type mean values for C57BL/6 mice.



Supplementary Figure 14 | Histology analyses of major organs for safety assessment of PINS.

Representative H&E sections of the main organs from C57BL/6 immunocompetent mice after bolus injection (250 mg/kg) or repeated injection (50 mg/kg/week, 5 injections) of PINS and sacrificed after selected time points ($n = 5$ for each group). At 250 mg/kg, microsteatosis was observed in the liver at earlier time points (day 1 and day 7), but recovered on day 28. Spleen, kidney and heart showed no abnormalities. For repeated injection, no abnormalities were observed in any of the main organs.

List of Supplementary Movies:**Movie S1**

Real-time tumour acidosis guided surgery (TAGS) of a HN5 head/neck tumour bearing mouse using the SPY Elite[®] camera. Movie on the left shows the white light surgery, and movie on the right shows TAGS. White light is not in interference with PINS imaging and can be left on during surgery. Visual inspection of tumour bed by eyes was not able to differentiate residual tumours from surrounding muscle tissue, but the residual tumours were successfully detected by PINS under the SPY Elite[®] camera and effectively removed.

Movie S2

Real-time TAGS of an orthotopic 4T1 breast tumour bearing mouse using the SPY Elite[®] camera. An orthotopic breast tumour in the mammary pad was imaged with high contrast in real-time by PINS and was removed accordingly.

Movie S3

Real-time TAGS of a small occult breast tumour bearing mouse using the SPY Elite[®] camera. Without looking at the fluorescence signal, the presence/location of the tumour nodule cannot be detected. With the help of PINS, a small occult disease was found beside the nipple and was removed with PINS guidance. Real-time color-coded image display was used during the surgery.