Supplementary Material

Image-guided surgery using near-infrared Turn-ON fluorescent nanoprobes for precise detection of tumor margins

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Supplementary Material and Methods

Synthesis and characterization of HPMA copolymer-Gly-Gly-Cy5 (P-GG-Cy5) non-cleavable (control) probe

Cy5-NHBoc was synthesized as previously described [1]. In order to activate the fluorophore, tert-butyloxycarbonyl protecting group (Boc) was removed from the reactive amine by mixing Cy5-NHBoc (6 mg, 0.00752 mmol) with trifluoroacetic acid (TFA) and dichloromethane (DCM) mixture (1:1) for 5 min following immediate high-vacuum evaporation. Next, Cy5-NH_2 fluorophore was conjugated with non-cleavable HPMA copolymer–Gly-Gly-*p*-nitrophenol (HPMA-GG-ONp) (11.4 mg, 0.0751 mmol) in 1 mL anhydrous *N*,*N*-Dimethylformamide (DMF) and 1 drop of trimethylamine ,Et_3N. The reaction mixture was stirred at room temperature (RT) in the dark for 12 h. The reaction progress was followed by High Pressure Liquid Chromatography (HPLC) (UltiMate® 3000 Nano LC systems, Dionex). At the end of the reaction, the solvent was removed partially by high vacuum and precipitated in cold ether. The precipitate was further washed with acetone and dried under vacuum. Purification of the conjugate and loading measurement was done in the same manner as describe in materials and methods for the synthesis of HPMA copolymer-GFLG-Cy5 (P-GFLG-Cy5) Turn-ON probe. Loading characterization showed that the conjugate had a 4.95 weight percent (wt%), 1.14 mol%, and 2.4 dye molecule per polymer chain.

Synthesis and characterization of PGA-Cy5 (PC) Always ON (control) probe

Poly-*L*-α-glutamic acid (PGA) was synthesized as previously described [2] by ring opening polymerization that started from α-*N*-carboxyanhydrides (NCA) of γ-benzyl-*L*-glutamate preparation. Neopentyl–NH_3BF_4 was used as an initiator. The PGA polymer obtained had a MW of 9959 Da, and PDI of 1.166, as characterized by multi-angle static light scattering (MALS) described later in this section. Cy5-NHBoc was conjugated directly to a PGA backbone coupling reaction. In order to activate the fluorophore, Boc protecting group was removed from the reactive amine by mixing Cy5-NHBoc (6.23 mg, 0.0078 mmol) with TFA and DCM mixture (1:1) for 5 min following immediate high-vacuum evaporation. Next, PGA (50 mg, 0.39 mmol) was dissolved in 1 mL anhydrous DMF and then transferred into the flask containing Cy5-NH_2 dissolved in 1 mL DMF. Then, the mixture was cooled in an ice bath. Next, Bis (2-oxo-3-

oxazolidinyl) phosphinic chloride (BOP-Cl) (4 mg, 0.015 mmol), 4-dimethylaminopyridine (DMAP) (4.4 mg, 0.036 mmol) and N,N-diisopropylethylamine (DIPEA) (2.5 µL, 0.014 mmol) were added. The reaction was mixed 4 h in an ice bath, and then ice was removed and the reaction continued for additional 24 h, under Argon ($Ar_{(g)}$) atmosphere. The reaction progress was followed by thin layer chromatography (TLC). At the end, 3 mL of 10% NaCl was added and the mixture was acidified to pH 2.5 by addition of 0.5 M HCl solution. The reaction was stirred for 1 h at RT and then the reaction solvent was removed by high vacuum. Purification of the conjugate and loading measurement was done in the same manner as describe in materials and methods for the synthesis of PGA-Cy5 (PC) Turn-ON probe. Cy5 loading characterization by free Cy5 calibration curve, resulted in 2.4 wt% and 0.56 mol%.

Dose-dependent activity of the cathepsin B (CTSB) inhibitor

Dose dependent activity of CTSB inhibitor, CA-074 Me, was evaluated by preincubation of various concentration of CA-074 Me (from 0.625 µM to 25 µM) with or without CTSB enzyme (0.2 U/mL) for 1 h in freshly prepared activity phosphate buffer (0.1 M, pH 5.5), containing 0.05 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM reduced glutathione (GSH). P-GFLG-Cy5 (10 µM Cy5-eq. concentration) was added to the reaction 1 h post incubation and further incubated for 116 h. The fluorescence of the degradation or the inhibited degradation of P-GFLG-Cy5 4.06 mol% was monitored using SpectraMax M5^e multi-detection reader.

CTSB activity assay and HPLC degradation follow-up

Cy5 enzymatically-directed release from the conjugates was studied *in vitro*, upon Turn-ON probes, P-GFLG-Cy5, PC and PGA-Cy5-Quencher (PCQ) (5 µM Cy5-eq. concentration) incubation at 37 °C with CTSB (0.56 U/mL) in freshly prepared activity phosphate buffer (0.1 M, pH 5.5), containing 0.05 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM reduced glutathione (GSH). The non-cleavable and Always ON probes, P-GG-Cy5 1.14 mol% and PC 0.56 mol%, respectively, served as control. All probes were incubated in the absence of CTSB at the same conditions. Free Cy5 release was monitored by measuring the change in the fluorescence intensity at sequential time points. The fluorescence measurements were carried out at excitation wavelength of 630 nm using SpectraMax M5^e multi-detection

reader. Samples (100 µL) were collected every 24 h (up to 70 h) and immediately analyzed. In addition, the Cy5 release was validated by high pressure liquid chromatography (HPLC) (UltiMate® 3000 Nano LC systems, Dionex). Reverse-phase high pressure liquid chromatography (RP-HPLC): Merck Millipore, C18 5u, 250x4.6 mm, LiChro column (gradient of 20% to 100% ACN in water, 0.1% TFA).

Labeling of intact cells with NIRF-ABP

4T1, MCF-7, DA3 and MDA-MB-231, B16-F10, WM115, A375, RMS cells (300,000 cell/well) and WM239A, 131/4-5B1, PD-MBM1, PD-MBM2 and PD-MBM3 cells (350,000 cell/well) were seeded in a 6-well plate 1 day before treatment. Cells were treated with 2 µM GB123, an activity based probe (ABP) labeled with Cy5 [3], for 4 h (0.1% DMSO final concentrations) by addition of the probe into growth medium. Cells were washed with phosphate-buffered saline (PBS) and lysed by addition of RIPA buffer (PBS, pH 7.4, 1% Tergitol-type NP-40 (nonyl phenoxypolyethoxylethanol), 0.1% SDS, and 0.5% sodium deoxycholate)). After centrifugation for 10 min (18500 rcf, 4 °C), the supernatant was collected and protein amount was quantified by Bradford assay. Amount equivalent to 35 µg protein was then separated by 12.5% SDS-PAGE. Labeled proteases from lysed cells, were visualized by scanning the gel with Typhoon FLA 9500 scanner at λ_{ex} /λ_{em} of 635/670 nm.

***In vitro* Turn-ON evaluation by human recombinant cathepsins**

Human recombinant (hr) cysteine cathepsins CTSL and CTSS were obtained commercially (R&D Systems). All hr cathepsins (100 µM) were incubated with P-GFLG-Cy5 and PCQ Turn-ON probes (10 µM Cy5-eq. concentration). Each protease was incubated in its suitable buffer and pH, according to the supplier recommendation. Hr CTSB, CTSS and CTSL reaction with the Turn-ON probes was carried out in acetate buffer (50 mM acetate, 4 mM DTT and 5 mM $MgCl_2$, pH 5.5), acetate buffer (50 mM acetate, 5 mM DTT and 250 mM NaCl, pH 4.5) and MES buffer (50 mM MES, 5 mM DTT, 1 mM EDTA, 0.005% w.t Brij-35, pH 6), respectively, for 24 h at 37 °C. As controls the Turn-ON probes were incubated in the different pH in the absence of the hr enzymes. Fluorescence intensity was measured using SpectraMax M5^e multi-detection reader at λ_{ex}/λ_{em} =630/670 nm.

In vitro Turn-ON evaluation in living cells

For Turn-ON evaluation in living cells, 131/4-5B1 human melanoma cells were incubated with 0.25 μM PCQ at 37 °C for 30 min. The treatments were washed three times with PBS and incubated with phenol red-free RPMI medium for 0, 0.5, 1 and 16 h. For evaluation of Turn-ON inhibition of cathepsins in living cells, cells were incubated with 2.5 μM GB111-NH_2, a cathepsins inhibitor [47], 1 h prior treatment with PCQ. Than PCQ was added to the growth medium and further incubated for 0.5 h. Cells were washed 3 times with PBS and detached from the petri dish by incubation with phenol-red free trypsin for 5 min. Then, 6 mL of PBS with 2% of FBS was added and the cells were centrifuged for 7 min at 200 rcf (4 °C). The cells were resuspended in 50 μL of PBS with 2% of FBS and analyzed by ImageStream®^{X} Mark II Imaging Flow Cytometer for the elevation in fluorescence signal of Cy5. The threshold for percentage (%) of cells with positive Cy5 signal (Cy5 +, marked in red, **Figure S5C**), was determined according to the fluorescence signal of the untreated (UT) cells' signal (gray).

Turn-ON probes stability in different media

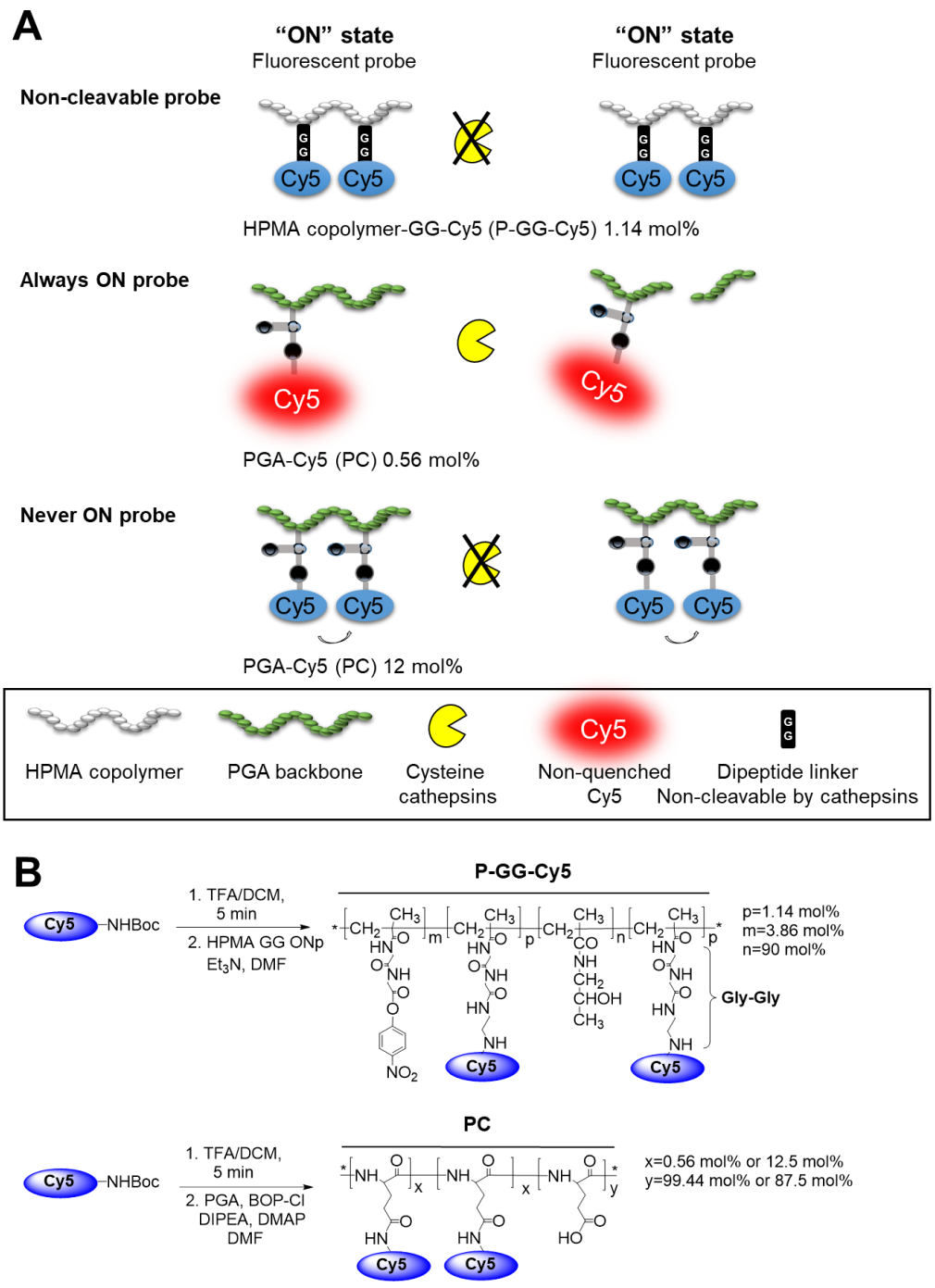
P-GFLG-Cy5 and PC were challenged in different media for 100 h, 37 ^{O}C. the media evaluated included: 10% and 50% mouse plasma in PBS, DMEM cell growth medium with 10% FBS and PBS (control), pH 7.4. The fluorescence intensity of 100 μL samples was measured by SpectraMax M5^e multi-detection reader at $\lambda_{ex}/\lambda_{em}$ =630/670 nm.

Turn-ON probes pharmacokinetics (PK) study

P-GFLG-Cy5 and PC conjugates were injected i.v. to 6-8-week-old female BALB/c mice (18–24 g) bearing 4T1 mammary adenocarcinoma and C57 mice bearing Ret melanoma tumors, respectively (3 mice per group). At predetermined intervals of 0, 10, 30, 60 and 180 min, blood samples (200 μL) were taken from the submandibular vein, and kept on ice. The samples were then centrifuged at 350 rcf for 10 min and 50 μL plasma supernatant samples were collected. The fluorescence signal of the Cy5 bearing conjugates inside the plasma was evaluated by SpectraMax M5^e multi-detection reader at $\lambda_{ex}/\lambda_{em}$ =630/670 nm. The concentration of probes in the plasma samples was calculated against the fluorescence calibration curve of each conjugate.

Fluorescence Optical Imaging

BALB/c mice bearing mammary adenocarcinoma 4T1 tumors (~100 mm^3) were injected intravenously (i.v.) with non-cleavable P-GG-Cy5 and Always ON PC 0.56 mol%, (10 μM; 200 μL saline, 5-6 mice per group). Fluorescence signal within tumor and within similar size area parallel to the tumor site, was assessed at different time points 4 h following injection in BALB/c mice using non-invasive imaging system CRI Maestro™. Multispectral image-cube were acquired through 650–800 nm spectral range in 10 nm steps using excitation (635 nm longpass) and emission (675 nm longpass) filter set. Mice auto-fluorescence and undesired background signals were eliminated by spectral analysis and linear unmixing algorithm.



Scheme S1: **Design and synthesis of non-cleavable HPMA copolymer-Gly-Gly-Cy5 (P-GG-Cy5), Always ON PGA-Cy5 (PC) and Never ON PC control probes. (A)** Schematic representation of Non-cleavable, Always ON and Never-ON control probes, where no change in fluorescence signal is observed upon incubation with cysteine cathepsins **(B)** Synthesis scheme of P-GG-Cy5 (upper panel) and PC with 2 different payloads of Cy5 (lower panel) control probes.

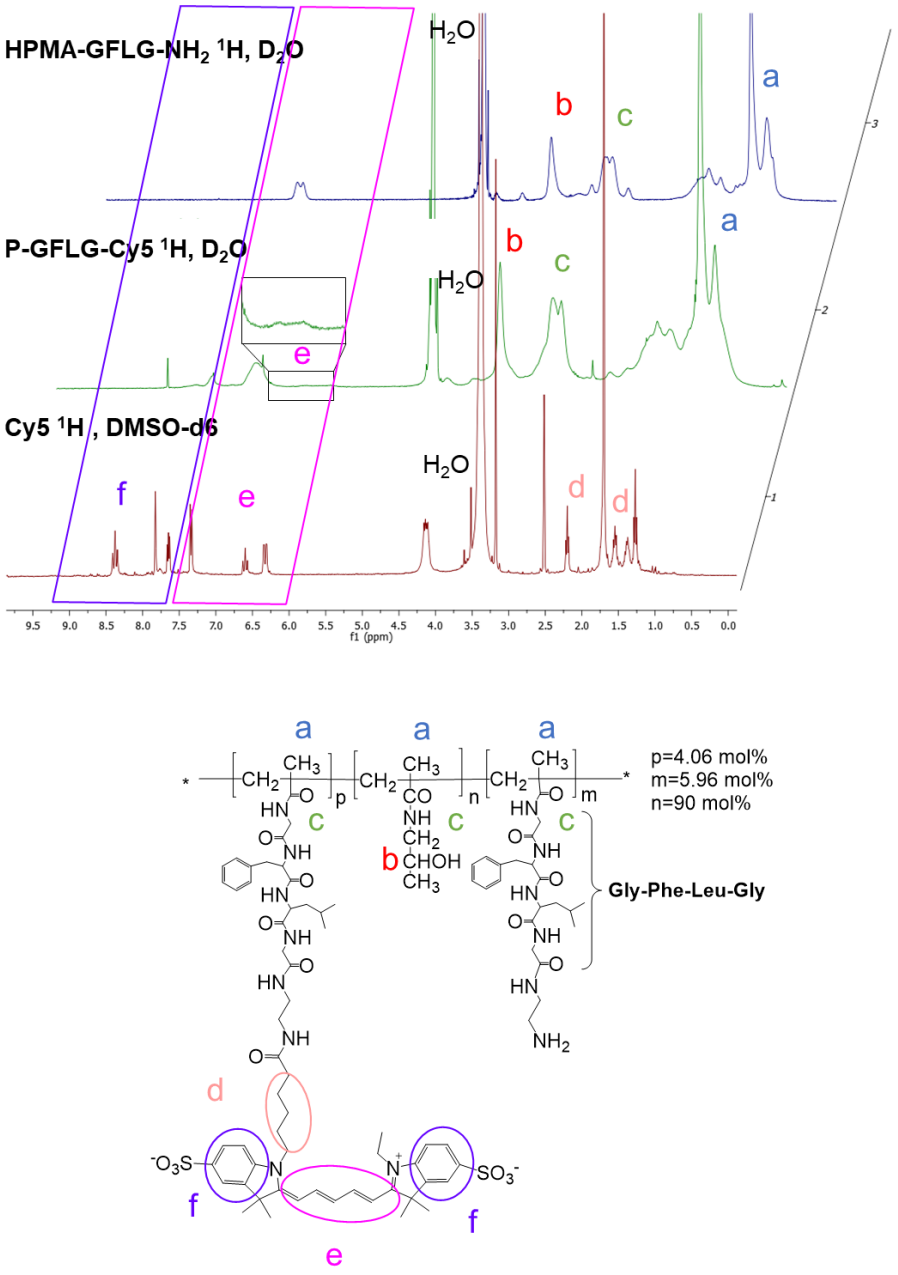
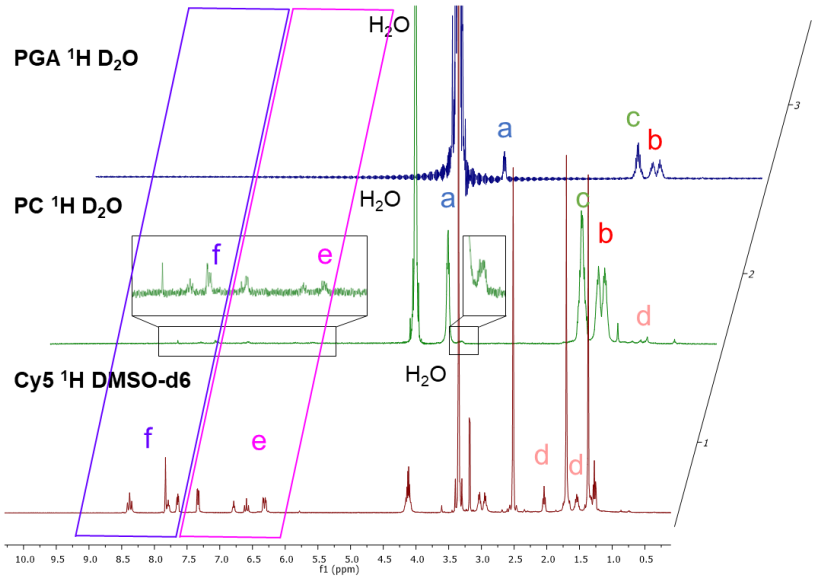


Figure S1: ^{1}H NMR (400 MHz) spectra of HPMA copolymer precursor, HPMA-GFLG-NH_2, (D_2O), P-GFLG-Cy5 (D_2O) and Cy5 COOH (DMSO-d6). HPMA-GFLG-NH_2: δ 7.41-6.98 (m, -NH), 3.78 (bs, 1H), 3.21-2.76 (m, 6H), 2.16-1.36 (m, 6H), 1.30-0.48 (m, 9H). P-GFLG-Cy5: δ 8.34 (s, 2H), 7.96 (bs, 2H), 7.71 (s, 2H), 7.41-6.98 (m, 2H), 6.44 (bs, 1H), 6.09 (bs, 2H), 3.78 (s, 1H), 3.21-2.76 (m, 6H), 2.16-1.36 (m, 6H), 1.30-0.48 (m, 9H). The relaxation delay (d1) time was 25 s. Cy5 COOH: δ 8.38 (t, J = 13.0 Hz, 2H), 7.83 (s, 2H), 7.65 (dd, J = 7.4, 6.1 Hz, 2H), 7.34 (d, J = 8.3 Hz, 2H), 6.60 (t, J = 12.3 Hz, 1H), 6.33 (dd, J = 13.8, 4.6 Hz, 2H), 4.13 (dd, J = 13.6, 6.8 Hz, 4H), 2.20 (t, J = 7.2 Hz, 2H), 1.70 (s, 12H), 1.54 (dd, J = 14.6, 7.4 Hz, 3H), 1.43 – 1.33 (m, 3H), 1.27 (t, J = 7.1 Hz, 3H).



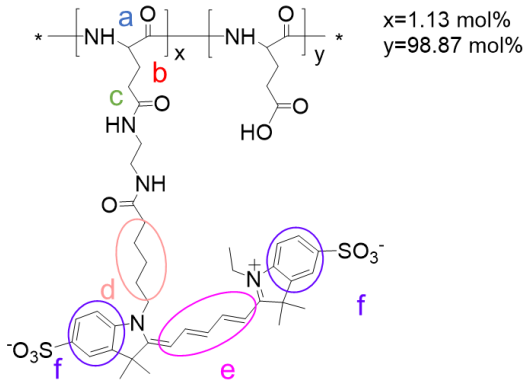


Figure S2: ^{1}H NMR (400 MHz) spectrum of PGA precursor (D_2O), PC (D_2O), and Cy5 (DMSO-d6). PGA: δ 4.1 (t, 1H), 2.05 (m, 2H), 1.87-1.59 (m, 2H). PC: ^{1}H NMR (400 MHz, D_2O) δ 8.33 (s, 2H), 7.98 (t, 2H), 7.74 (m, 2H), 7.26 (d, 2H), 6.53 (m, 1H), 6.28 (m, 2H), 4.30 – 4.13 (m, 1H), 4.09–3.93 (m, 4H), 2.25-2.04 (m, 2H), 1.99-1.73 (m, 2H) 1.87-1.59 (m, 6H). The relaxation delay (d1) time was 3 s. Cy5 amine: ^{1}H NMR (400 MHz, DMSO) δ 8.38 (t, J = 13.0 Hz, 2H), 7.83 (t, J = 1.3 Hz, 2H), 7.79 (t, J = 5.5 Hz, 1H), 7.65 (dd, J = 8.2, 3.7 Hz, 2H), 7.33 (dd, J = 8.3, 3.2 Hz, 2H), 6.79 (t, J = 5.4 Hz, 1H), 6.59 (t, J = 12.3 Hz, 1H), 6.32 (dd, J = 13.8, 4.2 Hz, 2H), 4.18 – 4.05 (m, 4H), 3.07-3.00 (m, 2H), 2.98-2.90 (m, 2H), 2.04 (t, J = 7.3 Hz, 2H), 1.70 (s, 12H), 1.60-1.48 (m, 2H), 1.37 (s, 9H), 1.42-1.30 (m, 2H), 1.27 (t, J = 12.2, 3H).

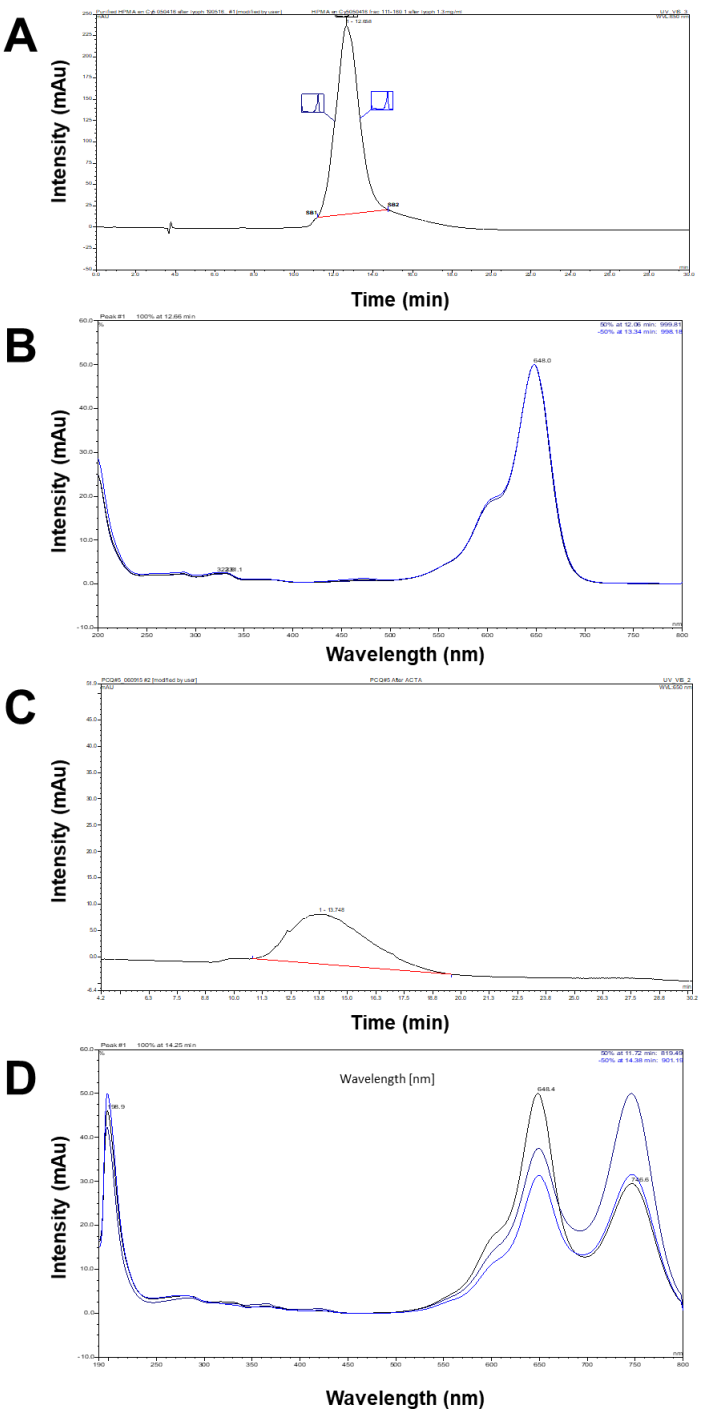


Figure S3: HPLC characterization of Turn-ON probes. (A) P-GFLG-Cy5 conjugate chromatogram. **(B)** Spectrum of the P-GFLG-Cy5 conjugate, with peaks at λ=600 and 650 nm, indicating Cy5 presence. **(C)** PGA-Cy5-Quencher (PCQ) conjugate chromatogram. **(D)** Spectrum of the PCQ conjugate, with peaks at λ=600, 650 and 750 nm, indicating Cy5 and quencher presence. 20-100% ACN:DDW with 0.01% TFA, flow rate: 1 mL/min.

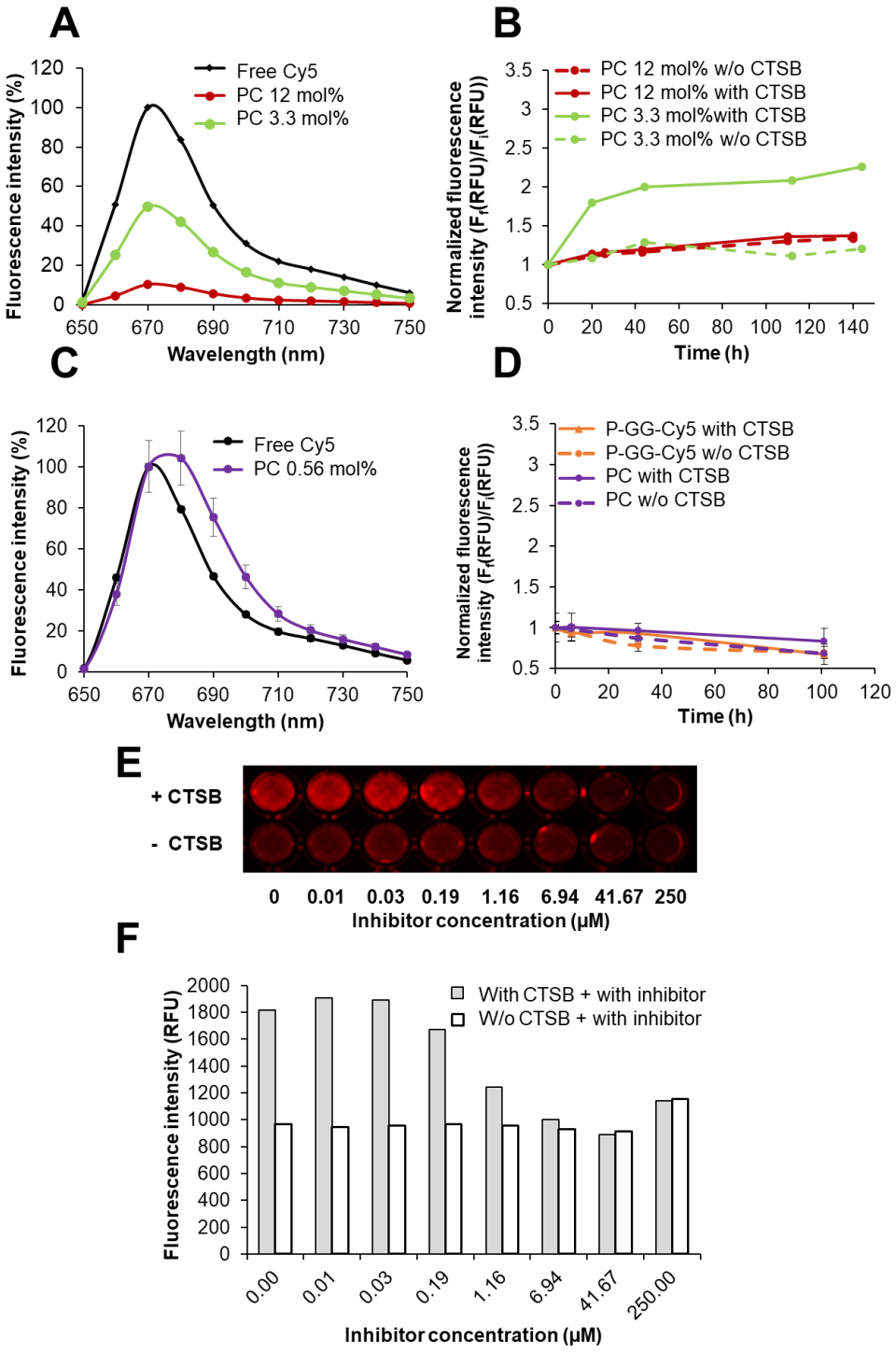
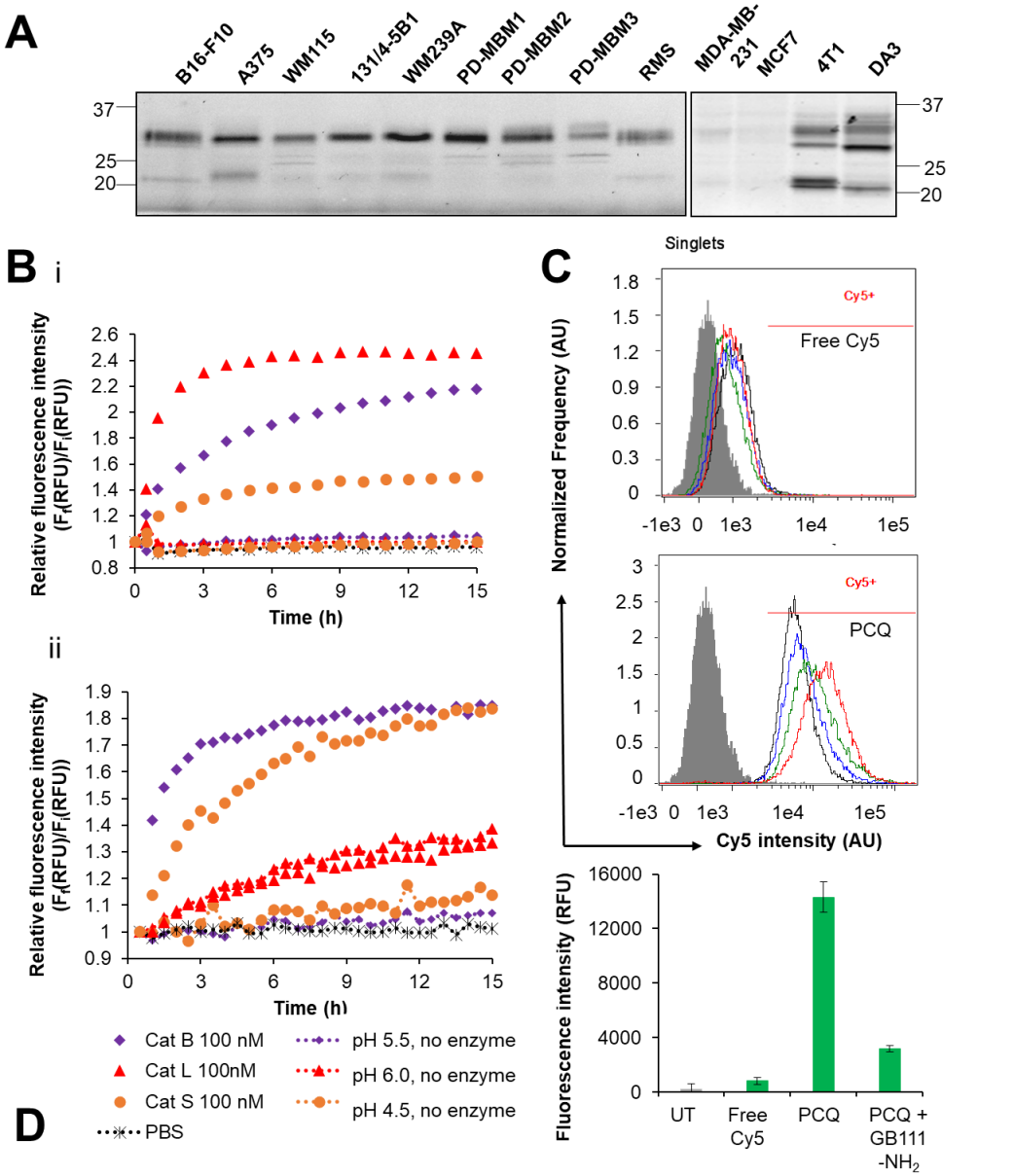


Figure S4: Turn-ON and quenching properties of different Turn-ON, Always ON, Never ON and non-cleavable probes. (A-C) Effect of Cy5 loading on the quenching efficiency and Turn ON ability. PC conjugates, bearing different Cy5 payload (red 12 mol% and green 3.3 mol%),were measured for fluorescence signal. The conjugate with higher payload of Cy5 had a higher quenching and a lower fluorescence intensity. **(B)** PC conjugates (red 12 mol%, green 3.3 mol%) incubation with (solid line) or without (dash type line) CTSB enzyme. The higher payload

of Cy5 (12 mol%, red) resulted in negligible elevation of the fluorescence signal, indicating a "Never ON" probe, probably due to steric hindrance. **(C)** PC conjugate bearing the lowest payload of 0.56 mol% (purple) showed no decrease in the fluorescence signal in comparison with free Cy5 (black), hence no quenching. **(D)** PC (purple 0.56 mol%) and non-cleavable P-GG-Cy5 (orange 1.14 mol%) conjugates incubation with (solid line) or without (dash type line) CTSB enzyme. The lower payload of Cy5 (0.56 mol%, purple) and the non-cleavable linker, Gly-Gly (GG), on the HPMA copolymer-based probe resulted in no elevation of the fluorescence signal, both indicating "Always ON" probes, the former is by introducing non-quenched Cy5 interactions due to a lower loading, and the last is by the addition of non-cleavable linker. All conjugates were measured for fluorescence signal at the same Cy5-eq. concentration (A and B 55 μM, C and D 5 μM) by SpectraMax M5^e multi-detection reader. Filter set: λ_{Ex}: 630 nm, filter: 665 nm, λ_{Em}: 670 nm. The data are presented as mean ± SD (n = 3). **(E-F)** Dose-dependent activity of the CTSB inhibitor. Upper panel represents a fluorescence imaging of Cy5 release from P-GFLG-Cy5 conjugate (4.06% loading; 10 mM Cy5-eq. concentration) by CTSB (0.2 U/mL) in the presence or absence of indicated inhibitor concentration (0-250 μM). A higher inhibition (lower signal intensity) was observed for increased inhibitor concentration 116 h post incubation. CTSB was not added in control wells. Lower panel represent the quantification of the fluorescence signal for each treatment. Data was imaged and acquired by CRI Maestro^{TM} (filter set: λ_{ex}: 635 nm, λ_{em}: 650-800 nm, filter: 675 nm) and SpectraMax M5^e multi-detection reader, respectively.



Treatment	UT	Free Cy5				PCQ			
Time post treatment removal (h)	0	0	0.5	1	16	0	0.5	1	16
Cells with positive Cy5 signal (%)	0.42	2.22	1.58	1.56	0.86	97.6	98.5	99.2	98.5
Mean Fluorescent signal (AU)	681.59	1149.2	1282.2	990.72	1011.86	7107	9353	13375	16576

Figure S5: Cathepsins activity and Turn-ON evaluation *in vitro* in intact and living cells. (A) Active cathepsins labeling with activity-based probe (ABP), GB123. Labeling of active cathepsins in murine melanoma B16-F10, RMS (Ret-mCherry), human A375, WM115, 131/4-5B1, WM239A and patient-derived melanoma brain metastases PD-MBM1, PD-MBM2, PD-MBM3 cell lines, murine mammary adenocarcinoma 4T1, DA3 and in the human breast cancer cell lines MCF7 and MDA-MB-231. Cells were treated with 2 µM GB123 for 4 hours (0.1%

DMSO final concentrations). Cells were then washed with PBS and lysed by addition of RIPA buffer, cell debris were pelleted and supernatant was collected. Amount equivalent to 35 µg protein (determine by Bradford assay) were then separated by 12.5% SDS-PAGE, and labeled proteases were visualized by fluorescence scanning of the gel for Cy5 signal with Typhoon scanner, FLA 9500 at 635/670 nm excitation/emission. The fluorescent bands between 20-37 kDa are characteristic for cathepsin enzymes as was previously demonstrated [4, 5]. **(B)** Activation of P-GFLG-Cy5 (i) and PCQ (ii) by human recombinant (hr) cysteine cathepsins (100 nM each in its suitable buffer): CTSL (red), CTSB (purple), CTSS (orange) in MES buffer (pH 6), acetate buffer (pH 5.5) and acetate buffer (pH 4.5), respectively. The incubation of the probes in the different buffers, suitable for each hr enzyme, (pH 6, 5.5, 4.5) or in PBS (pH 7.4) but without hr cathepsins is indicated by dashed lines at the corresponding colors. **(C)** Fluorescence signal histograms of primary melanoma 131/4-5B1 cells treated with free Cy5 (0.25 µM, upper panel) and PCQ Turn-ON probe (0.25 µM Cy5-eq. concentration, middle panel). 131/4-5B1 melanoma cells were incubated with Cy5 or PCQ for 0.5 h. Then, the treatments were removed, washed with PBS and replaced with the cells' growth medium for further incubation. Live cells were monitored at 0 (black), 0.5 (blue), 1 h (green) and 16 h (red) following treatment removal. Untreated cells (gray) served as control and the threshold for Cy5 signal positive cells was determined according to them. Lower panel: inhibition of PCQ Turn-ON in living cells by GB111-NH_2, cathepsins' inhibitor. GB111-NH_2 (2.5 µM) was added 1 h prior to treatment with PCQ (0.25 µM Cy5-eq. concentration, 0.5 h incubation). Fluorescence signal from living cells treated with PCQ + GB111-NH_2 or with PCQ alone was evaluated 1 h post treatment removal. Untreated cells and free Cy5 served as control. Data represent mean ± SD (n=3). **(D)** Summary table of percentage of cells with positive Cy5 signal and mean fluorescence signal calculated from histograms. The summary table and histograms show elevation in the fluorescence signal over time (up to 16 h), indicating the PCQ probe's Turn-ON ability when introduced to 131/4-5B1 cells, which had highly active cathepsins. In addition, when cathepsins' inhibitor GB111-NH_2 was added, the increase in the fluorescence intensity was almost abolished, indicating an efficient inhibition of the probe's activation. Fluorescence intensity of free Cy5 remained stably low at all time points, due to its poor cellular internalization probably arising from its negative charge. Images and analysis were obtained using ImageStream®^{X} Mark II Imaging Flow Cytometer.

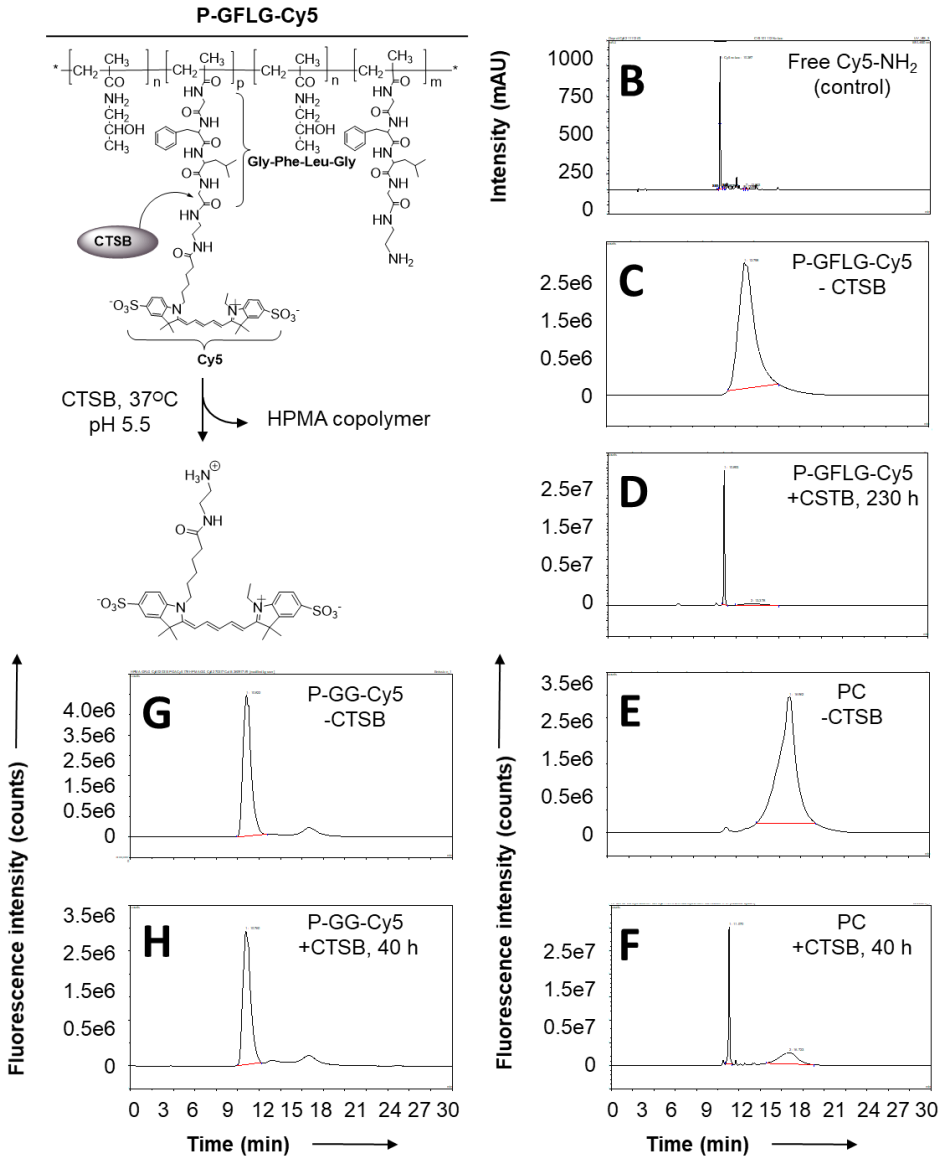


Figure S6: HPLC analysis (λ_{ab} = 650 nm, $\lambda_{ex}/\lambda_{em}$ = 630/670 nm) of Turn-ON probes degradation by reaction with CTSB. (A) Chemical structure and cleavage mechanism by CTSB of P-GFLG-Cy5 conjugate. **(B)** Free Cy5-NH_2 (control, retention time 10.38 min) obtained by removal of Boc protecting group from Cy5-NHBoc (1:1 DCM:TFA for 5 min). **(C)** P-GFLG-Cy5 with no CTSB (retention time 12.79 min). **(D)** Cy5 release (retention time 10.89 min) form P-GFLG-Cy5 (retention time 13.3 min) by CTSB enzyme, 64 h post incubation at pH 5.5, 37 ^{O}C. **(E)** PC with no CTSB (retention time 16.92 min). **(F)** Release of Cy5 form PC conjugate by CTSB enzyme, 40 h post incubation at pH 6, 37 ^{O}C (retention times are 11.07 and 16.72, respectively). **(G and H)** Always ON (control) probe, bearing non-cleavable linker Gly-Gly (GG), P-GG-Cy5 in the absence **(G)** or presence (retention times are 10.82 and 10.78 min, respectively) **(H)** of CTSB enzyme, 40 h post incubation at pH 6, 37 ^{O}C. No degradation of Cy5 was observed by HPLC analysis. Y-axes represent fluorescence intensity (counts, $\lambda_{ex}/\lambda_{em}$ = 630/670 nm) or absorbance intensity (Intensity mAU, λ_{ab} = 650 nm). HPLC gradient – 20-100 ACN in DDW).

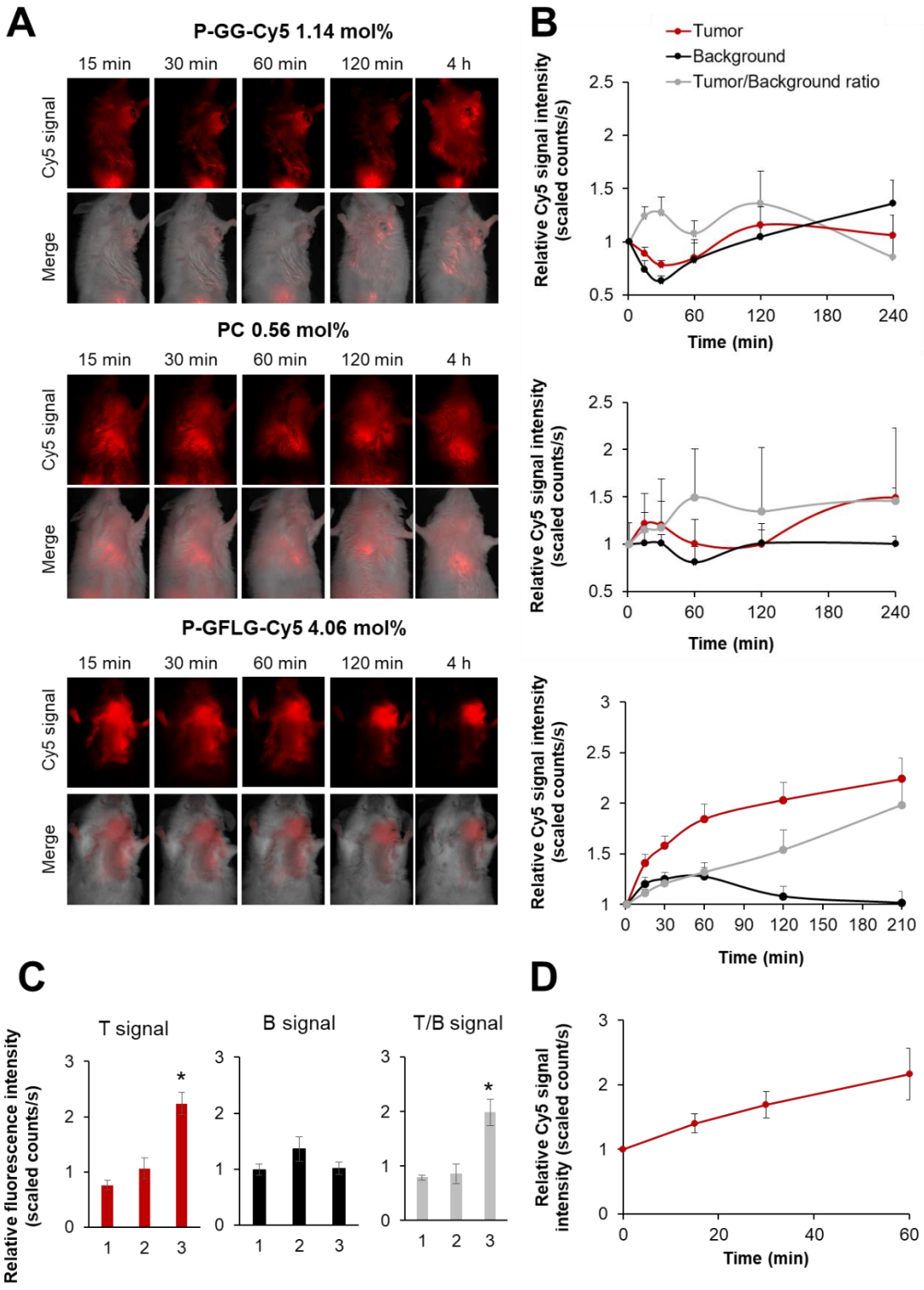


Figure S7: Comparison of tumor-to-background signal between Always ON and Turn-ON probes. (A) Representation of kinetic follow-up post i.v. administration of Always ON probes (P-GG-Cy5 and PC 0.56 mol%) and Turn-ON probe (P-GFLG-Cy5 4.06 mol%) to BALB/c mice

bearing 4T1 mammary adenocarcinoma tumors (n=5 and 9). **(B)** Relative fluorescence signal up to 4 h post i.v. administration from the tumor (red), background (black) and tumor-to-background ratio (gray) of the conjugates represented at panel A - P-GG-Cy5 (top), PC 0.56 mol% (middle) and P-GFLG-Cy5 4.06 mol% (bottom). **(C)** Relative fluorescence signal of the control probes, P-GG-Cy5 (1), PC 0.56 mol% (2) and Turn-ON probe P-GFLG-Cy5 4.06 mol% (3), from tumor (T, red), Background (B, black), and tumor-to-background (T/B, gray) 4 h post i.v. administration. (* One way ANOVA, $p<0.001$). **(D)** Fluorescence signal elevation 60 min following i.t injection of Turn-ON probe, P-GFLG-Cy5 4.06 mol%, (30 µL, 100 µM Cy5-eq. concentration) in 4T1 adenocarcinoma tumors bearing mice (n=4). All probes were i.v. administered (200 µL, 10 µM Cy5-eq. concentration) unless otherwise stated. Data represent mean ± SD.

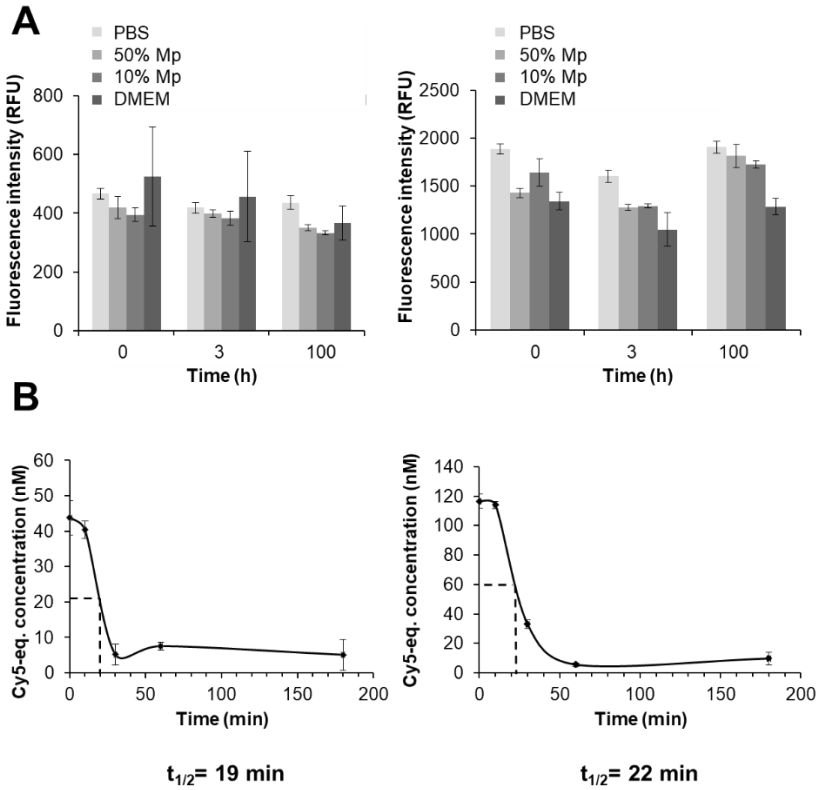


Figure S8: *In vitro* stability and Pharmacokinetics of P-GFLG-Cy5 and PC conjugates. (A) P-GFLG-Cy5 (left) and PC (right) were incubated with PBS pH 7.4, 50% mouse plasma (Mp) in PBS, 10% Mp and cell growth media (DMEM supplemented with 10% FBS) for 100 h in 37 ^{O}C. The fluorescence intensity was measured using SpectraMax M5^{e} multi-detection reader, and a negligible elevation in the signal was observed. **(B)** Pharmacokinetic measurements for P-GFLG-Cy5 (left) and PC (right). The Turn-ON probes were injected i.v. to mice bearing 4T1 mammary adenocarcinoma or Ret melanoma primary tumors. Blood samples were taken at 0, 10, 30, 60 and 180 min. The Cy5-eq. concentration in plasma samples was calculated according to each Cy5-bearing conjugate fluorescence calibration curve in PBS. The $t_{1/2}$ was 19 and 22 min for P-GFLG-Cy5 and PC, respectively. Data represent mean ± SD (n=3).

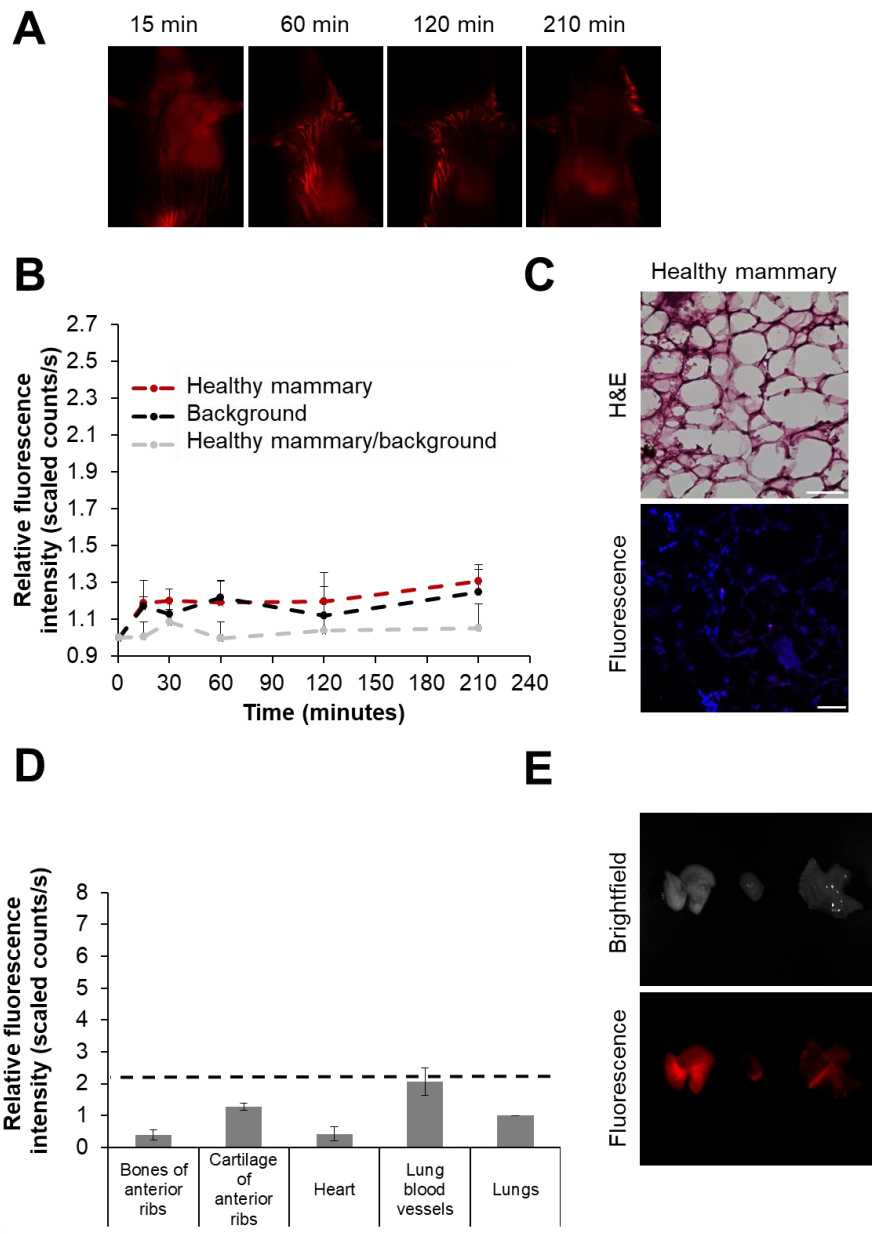


Figure S9: I.v. administration of P-GFLG-Cy5 (10 µM, 200 µL Cy5-eq. concentration) to healthy mice: (A) Kinetic follow up post i.v. injection. **(B)** Signal quantification from the same areas taken from 4T1 mammary adenocarcinoma bearing mice. Data represented as mean ± SD (n=6). **(C)** Representative healthy mammary fat pad excised from healthy mice post i.v. administration of P-GFLG-Cy5 probe. H&E analysis of healthy mammary fat pad, below: confocal image of the adjacent section. Red: Cy5 signal. Blue: DAPI. White scale bar indicates 50 µm. **(D)** Uptake of P-GFLG-Cy5 to healthy organs, 4 hours post i.v. administration. Data represented as mean ± SD (n=3). **(E)** Brightfield and Cy5 fluorescence signal of a representative CRI maestro image of healthy organs (from left to right: lungs, heart and rib cage).

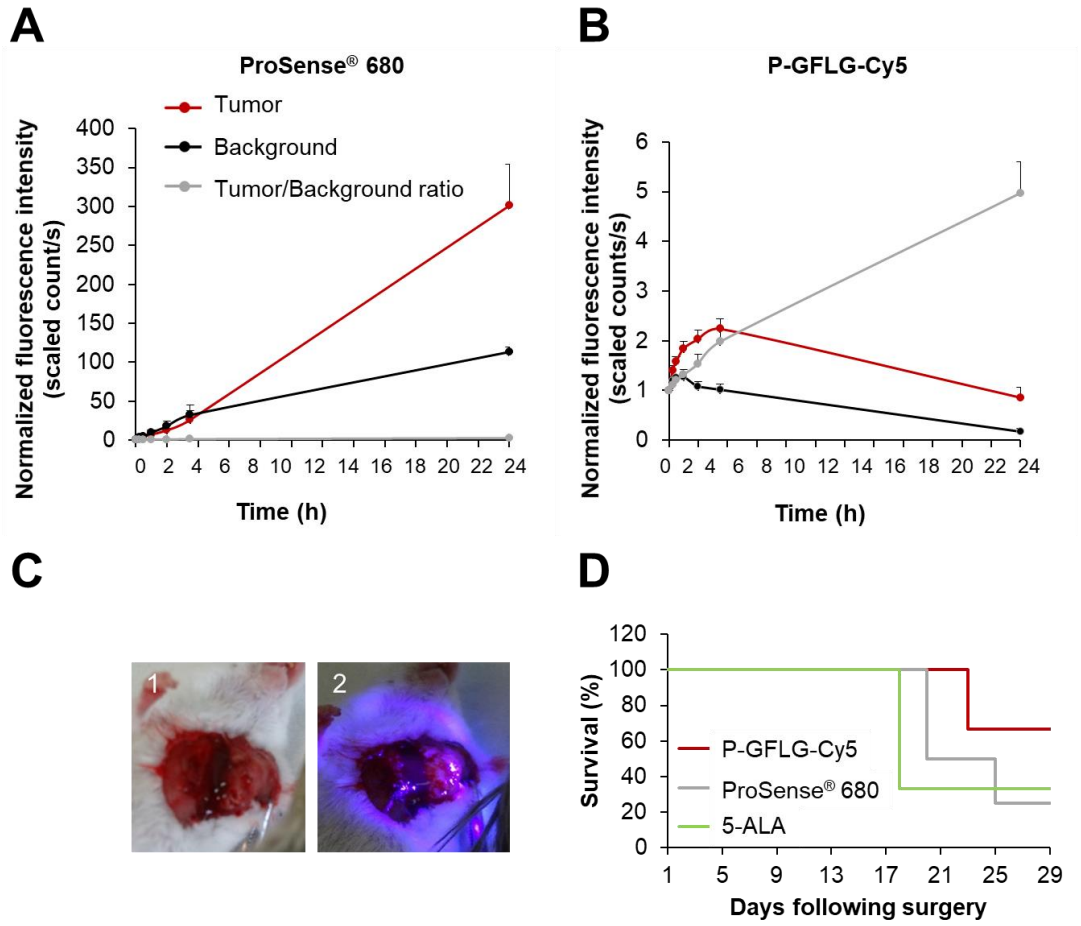


Figure S10: Comparison between P-GFLG-Cy5, ProSense® 680 and 5-aminolevulinic acid (5-ALA) as imaging agents for surgical procedures guidance. (A-B) Normalized fluorescence signal up to 24 h post i.v. administration from the tumor (red), background (black) and tumor-to-background ratio (gray) of 2 nmol in 150 µL (PBS pH 7.4), ProSense® 680 **(A)** and 10 µM in 200 µL saline, P-GFLG-Cy5 **(B)**. **C.** Surgery performed by 5-ALA guidance. **1.** Color image **2.** The visible fluorescence imaging of the tumor during the surgical resection. 5-ALA (0.1 mg/g) was administered per os to BALB/c mice bearing 4T1 adenocarcinoma mammary tumors. Image guided surgery was started 4 h post administration, under blue light illumination (OmniCure LX400 LED lamp, λ_{Ex} 400 nm). **D.** Kaplan–Meier survival curve. Overall survival (OS) after surgery *versus* time (days) of 4T1 mammary adenocarcinoma bearing mice that underwent surgeries under the guidance of P-GFLG-Cy5 (n=3, red), ProSense® 680 (n=4, gray) and 5-ALA (n=3, green).

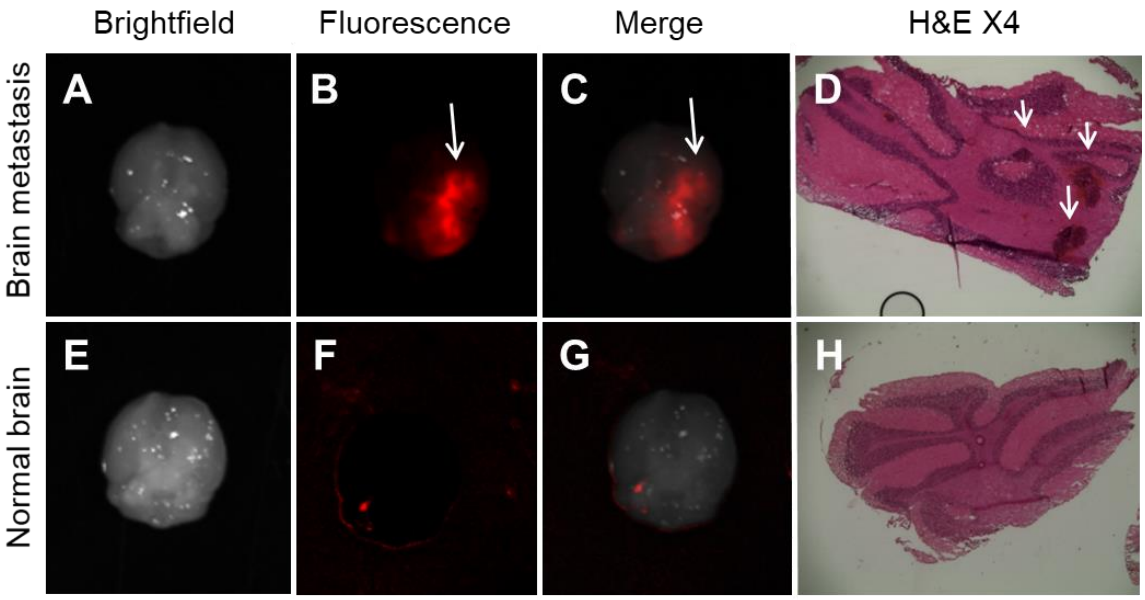


Figure S11: Brain metastases identification post i.v. administration of P-GFLG-Cy5 (200 µL, 10 µM Cy5-eq. concentration): Brightfield **(A)**, visualization of P-GFLG-Cy5 fluorescence signal (red) in brain tissue suspected metastases, using a CRI Maestro imaging system **(B)** and merge of brightfield and fluorescence imaging **(C)**. **D.** Histology of the tumor and metastases in the brain (marked in white arrows). Red signal that was obtained in the brain and suspected as metastases was confirmed by the H&E staining. Healthy brain served as control, and was examined by i.v. administration of P-GFLG-Cy5 as well: Brightfield **(E)**, no visualization of P-GFLG-Cy5 signal (red) in normal brain tissue, using a CRI Maestro imaging system **(F)** and merge of brightfield and fluorescence imaging **(G)**. **H.** H&E staining of normal healthy brain.

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