Electronic Supplementary Information (ESI)

Rapid, sensitive, and in-solution screening of peptide probes for targeted imaging of live cancer cells based on peptide recognition-induced emission

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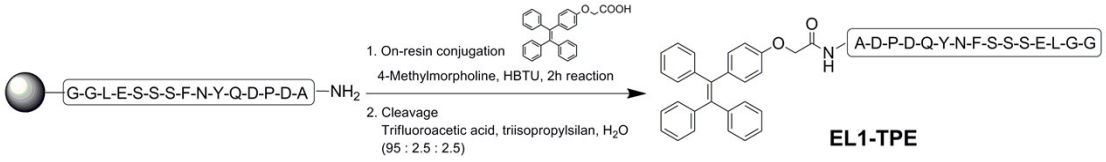
1. Experimental

Materials. (Fmoc)-protected L-amino acids, Fmoc-Gly-Wang resin and Fmoc-Ser(But)-Wang resin were provided by Advanced ChemTech (Louisville, USA). 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 2-(1H-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from GL Biochem (Shanghai, China). *N*,*N*-dimethylformamide (DMF) was purchased from BeiJing BoMaiJie Technology (Beijing, China). Acetonitrile (HPLC grade) was from Fisher Chemical (USA). Fmoc- propargyl-gly-OH, triisopropylsilane (TIPS) and trifluoroacetic acid (TFA, HPLC grade) were purchased from Aladdin (Shanghai, China). 4-Methylmorpholine, hexahydropyridine, ethanol, methanol, dichloromethane, hydrogen peroxide, sulfuric acid, formic acid, dimethyl sulfoxide (DMSO) of analytical grade and cupric sulfate were provided by Beijing Chemical Factory (Beijing, China). Antisense peptides used for solution experiments were synthesized and purified by commercial companies. AP1-AP4 were from ChinaPeptides (Shanghai, China) and AP5-AP8 were from SciLight Biotechnology (Beijing, China). Milli Q water purification system (Millipore, Bedford, MA, USA) was used to prepare ultra-pure water. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), IR-780 iodide, glytaraldehyde solution (50%) and cysteamine hydrochloride was obtained from Sigma-Aldrich (USA). LysoTracker® Green DND-26 were from life technologies (Thermo Fisher Scientific, USA). DAPI was obtained from Solarbio (Beijing, China). 3-chloropropylamine hydrochloride was purchased from J&K Scientific Ltd. (Beijing, China). Sodium azide was obtained from Merck Schuchardt (Hohenbrunn, Germany). Sodium cyanoborohydride was purchased from Alfa Aesar (Thermo Fisher Scientific, USA).

Apparent. The products were purified on a Shimadzu HPLC system with a UV-Vis detector (Kyoto, Japan). Compounds were analyzed and characterized with a ThermoFisher Ultimate 3000 UHPLC system coupled with an Ultimate 2000-LCQ Fleet mass spectrometer (CA, USA). The fluorescence spectra were recorded on a Hitachi F-4600 spectrometer with a Xe lamp as the excitation source (Tokyo, Japan). Microplate assays were measured on an Enspire Multimode Plate Reader (PerkinElmer, Cleveland, USA) and the 96-well black microplates were purchased from Corning (CONSTAR®, USA). Surface plasmon resonance (SPR) assays were recorded on PlexArray HT (PLEXERA, Washington, USA). The results of MTT assays were read on a SpectraMax M5 (Molecular Devices, USA). Confocal fluorescence imaging experiments were performed on a Confocal Laser Scanning Microscope (CLSM) FV1000-IX81 (Olympus, Japan).

Synthesis of EL1-TPE. 2-(4-(1,2,2-triphenylvinyl)phenoxy)acetic acid (TPE-COOH) was synthesized as previously described. The target peptide EL1 was synthesized manually by FMOC solid phase peptide synthesis (SPPS) approach. Before cleavage, TPE-COOH was conjugated to Wang resin-EL1 through the condensation of its carboxyl group and amino terminal of EL1. Afterward, the conjugate EL1-TPE was deprotected and cleaved from the resin (Scheme S1). The crude product was obtained as white solid by precipitation with diethyl ether. After HPLC purification, EL1-TPE was characterized by UHPLC-MS/MS (Figure S1).



Scheme S1. The synthesis procedure of EL1-TPE.

Fluorescence spectra measurements. A stock solution of EL1-TPE was prepared by dissolving the solid with DMSO to a concentration of 2.5 mM. To examine the binding between EL1-TPE and different peptides, the stock solutions of each peptide were prepared with phosphate buffer, respectively. Then, 0.8 μL of EL1-TPE stock solution was mixed with each peptide respectively, followed by the dilution with phosphate buffer to a final volume of 200 μL. The final concentration of the peptide was 20 μM, and the final fraction of DMSO in the mixture was 0.4%. After the incubation at room temperature for different interval, the fluorescence spectra were recorded; the excitation wavelength was 330 nm and the emission was collected from 340 to 650 nm.

Affinity screening assay in 96-well microplates. For the microplate assays, the procedure was similar to the fluorescence spectra measurements. In brief, 1.2 μL of EL1-TPE stock solution was mixed with each peptide respectively, followed by the dilution with phosphate buffer to a final volume of 300 μL. The final concentration of each peptide candidate was 20 μM, and the final fraction of DMSO in the mixture was 0.4%. After the incubation at room temperature, the 96-well plates were scanned by an Enspire Multimode Plate Reader. The excitation wavelength was 330 nm and the emission was at the wavelength of 470 nm. The measure mode is top-read.

SPR sensing. Sensor modification was carried out on bare gold-coated SPR chips (Nanocapture gold chips, with a gold layer of 47.5 nm thickness). The chip was pre-treated

with Piranha, water, and ethanol. After air dry, the chip was immersed into 20 mM cysteamine and kept at 4 ^{o}C in the dark overnight. Then the chip was washed and dried followed by the treatment with glutaraldehyde (pH 7.4, 2.5% by volume) at 40 ^{o}C for 4 hours. After being washed with water and dried, peptides AP1-AP8 (5 mg/mL each, in water) were printed on the chip respectively and left for conjugation overnight. After wash, 1% $NaCNBH_3$ was used for schiff base reduction. 1× PBS, 0.1× PBS and water were then used to wash the chip. Finally, the chip was blocked with ethanolamine (1 M in water, pH 7.4) for 1 hour. SPR analysis was operated at a flow rate of 2 μL/s. The chip surface was first balanced with PBS to obtain a baseline. EL1-TPE solutions at different concentrations (5 μg/mL, 10 μg/mL, 20 μg/mL, 40 μg/mL and 100 μg/mL) were injected respectively for the interaction with immobilized peptides. Real-time binding signals were recorded and analysed by PlexArray HT software.

Cells. Human Liver Hepatocellular Carcinoma cells (HepG2 cells) and Human Embryonic Kidney 293 cells (HEK293 cells) were from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). High glucose DMEM was obtained from HyClone (USA). Fetal bovine serum (FBS), penicilli,streptomycin and 0.25% Trypsin-EDTA were purchased from gibco (USA). Cells were cultured at 37 ^{o}C and 5% CO_2 in high-glucose DMEM supplemented with 10% FBS and 1% penicillin, streptomycin.

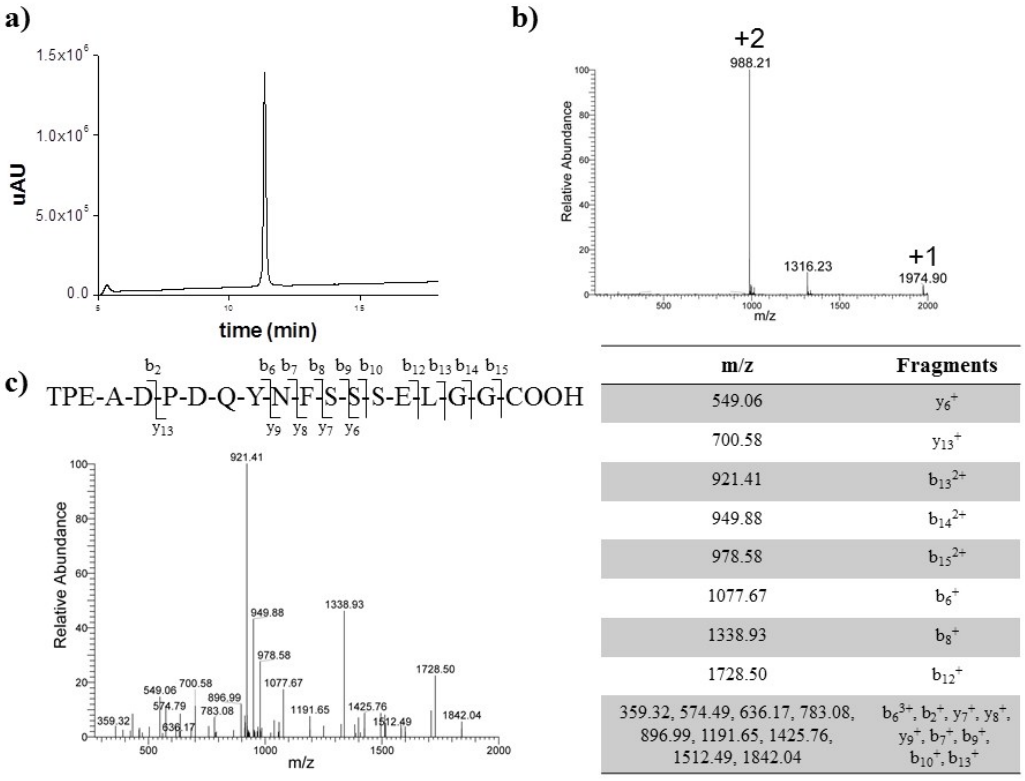
Cell imaging. The cells (approximately 1.0×10^5 mL^{-1}) were seeded and cultured overnight in 35 mm glass-bottomed dishes for adhesion. After removal the culture medium, the cells were treated with AP2-Cy7 or AP5-Cy7 (200 μL, 25 μM in medium with 0.5% DMSO) respectively for 1 hour in an incubator (37 ^{o}C, 5% CO_2). After incubation, the cells were carefully washed three times with PBS. For co-staining with DAPI, the peptide probe-loaded cells were further treated with DAPI for another 10 minutes. For co-staining with LysoTracker, the peptide probe-loaded cells were further treated with LysoTracker Green for another 30 minutes. The cells were carefully washed with PBS and subjected for observation.

Fluorescence imaging experiments were performed on an FV 1000-IX81 CLSM (Olympus, Japan). The objective used for imaging was a UPLSAPO 100× oil-immersion objective (Olympus). Peptide probes were excited by an FV10-LD 635 nm Laser Head, and the fluorescence was collected with a band-pass filter within the range of 655-755 nm. DAPI was excited by a 50 mw FV5-LD405-2 405 nm Laser Head, and the fluorescence was collected with a band-pass filter within the range of 425-525 nm. LysoTracker Green was

excited by an FV5-LAMAR 488 nm Laser Head, and the fluorescence was collected with a band-pass filter within the range of 500-600 nm. Imaging processing and analysis were performed on Olympus software (FV10-ASW).

MTT assay. Cell viability was evaluated by standard MTT assay. Cells were seeded in 96-well culture plates (approximately 5000 cells/well) and incubated overnight in culture medium. After cell adhesion, the medium in each well was replaced with 100 μL peptide solutions (25 μM, 50 μM and 100 μM in culture medium). After incubation for 48 hours, the peptide solutions were carefully removed, and 100 μL MTT solution (0.5 mg/mL, freshly prepared in DMEM) was added into each well. After incubation for 4 hours, the MTT solution was removed and 150 μL DMSO was added to the wells to dissolve the formazan crystals. The plates were shaken for 10 minutes and then the absorbance values at 490 nm were read by a microplate reader. The cell viability was calculated from the following equation: Cell viability = $A/A_0 \times 100\%$, where A is the absorbance values of the experiment group and A_0 is the absorbance values from the cells cultured in serum-supplemented medium without any treatment.

2. HPLC analysis and MS characterization of EL1-TPE



m/z	Fragments
549.06	y_6^+
700.58	y_{13}^+
921.41	b_{13}^{2+}
949.88	b_{14}^{2+}
978.58	b_{15}^{2+}
1077.67	b_6^+
1338.93	b_8^+
1728.50	b_{12}^+
359.32, 574.49, 636.17, 783.08, 896.99, 1191.65, 1425.76, 1512.49, 1842.04	b_6^{3+}, b_2^+, y_7^+, y_8^+, y_9^+, b_7^+, b_9^+, b_{10}^+, b_{13}^+

Figure S1. (a) HPLC analysis of EL1-TPE on a Welch C_{18} column (100 × 2.1 mm) monitored at the wavelength of 220nm. Retention time of EL1-TPE: 11.35 min. Gradient: 0-3-3.01-18min, 30%B-30%B-40%B-80%B (A: H_2O containing 0.1% fomic acid, B: acetonitrile containing 0.1% fomic acid). (b) ESI-MS characterization of EL1-TPE (ESI positive, m/z $(M+H)^+$, calcd:1974.86; found: 1974.90, m/z $[(M+2H)/2]^{2+}$ calcd: 987.93; found: 988.21). (c) ESI-MS/MS spectra of the peak of 988.21 in (b) and the information of the detected fragments.

3. Peptide design and characterization

The degeneracy of antisense peptides originated from the degeneracy of genetic codes allows the directional design of peptide libraries with condensed content. Two groups of peptide candidates were tailored according to the complementary DNA sequence of EL1 and the degeneracy of antisense peptides (Table 1 in the main text). The degeneracy of serine (S) in EL1 was used because the four antisense amino acids (glycine, arginine, alanine and threonine) of serine (S) have high diversity in chemical structures and properties. Glycine (G) is the simplest amino acid with no side chain; arginine (R) has a basic guanidine side chain; alanine (A) with a methyl group is a hydrophobic residue; the hydroxyl side chain in threonine (T) makes it a hydrophilic residue, providing the capability to form hydrogen bonds. Therefore, by using the degeneracy of serine residue, peptides with various degrees of binding capacity can be designed.

It is noticed that there are three adjacent serine residues at position 9-11 in EL1. In order to examine the feasibility and sensitivity of the AIE-based screening method, peptide candidates were designed in two groups with three-residue and one-residue changes respectively (Table 1 in the main text). In the first group, AP1-AP4 were designed by using the degeneracy of all three serine residues in EL1. The three serine residues at positions 9-11 in EL1, were respectively replace by triple G, R, A or T in AP1-AP4. For AP5-AP8 in the second group, they are identical in fourteen residues, which are direct read-out of the complementary codons of EL1 except that the residue corresponding to the position 9 serine in EL1 is replaced by antisense amino acids G, R, A or T respectively in each peptide. By screening these two groups of peptides with different level of diversity, the effectiveness of the method can be evaluated. The purity of all peptide candidates was verified with HPLC and satisfied the study of their affinity interactions with target sequence (Figure S2, Figure S3 and Table S1).

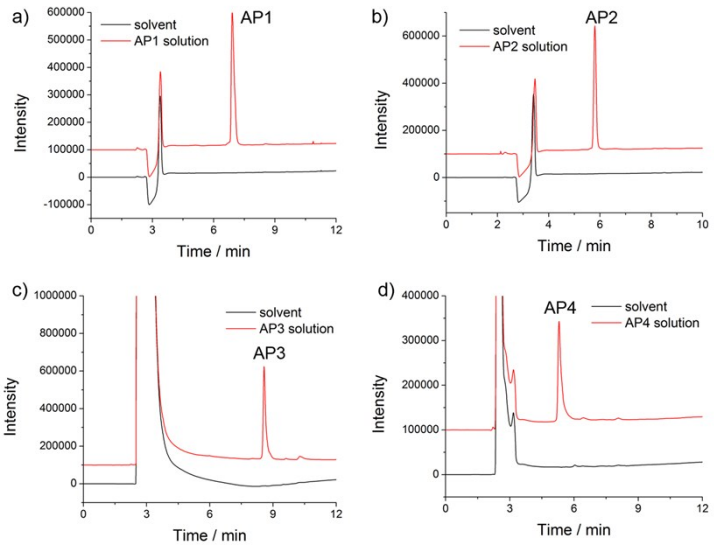


Figure S2. HPLC chromatograms of AP1-AP4. HPLC separation was performed on a Diamonsil C18(2) column (250 × 4.6 mm) monitored at the wavelength of 220 nm. Mobile phases: A: H_2O containing 0.1% fomic acid, B: acetonitrile containing 0.1% fomic acid. (a) AP1: Gradient: 0-11 min, 20%B-50%B; (b) AP2: Gradient: 0-11 min, 20%B-50%B; (c) AP3: Gradient: 0-13 min, 15%B-60%B; (d) AP4: Gradient: 0-10 min, 25%B-55%B.

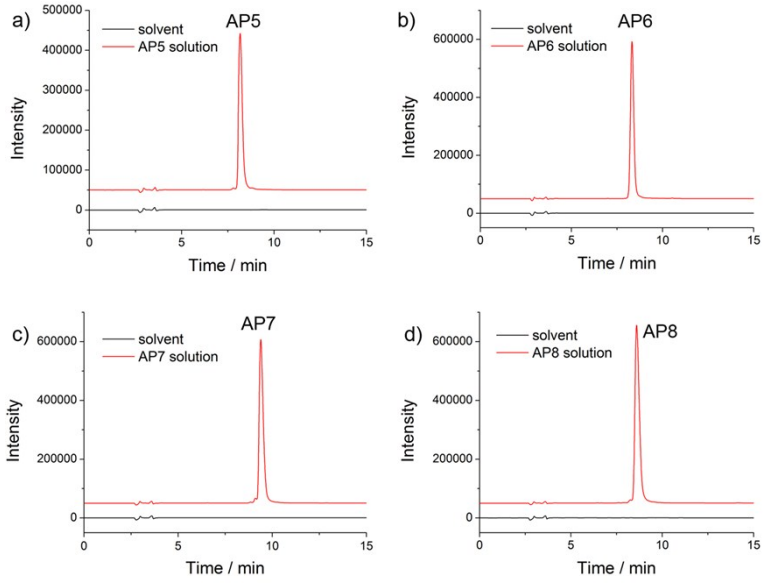


Figure S3. HPLC chromatograms of AP5-AP8. HPLC separation was performed on a Diamonsil C18(2) column (250 × 4.6 mm) monitored at the wavelength of 220 nm. Mobile phases: A: H_2O containing 0.1% fomic acid, B: acetonitrile containing 0.1% fomic acid. (a) AP5: Gradient: 0-15 min, 22%B-37%B; (b) AP6: Gradient: 0-15 min, 20%B-35%B; (c) AP7: Gradient: 0-15 min, 22%B-37%B; (d) AP8: Gradient: 0-15 min, 22%B-37%B.

Table S1. Purities of peptides AP1-AP8.

Peptide	Purity
AP1	99%
AP2	99%
AP3	97%
AP4	98%
AP5	99%
AP6	99%
AP7	99%
AP8	99%

4. SPR sensing the binding of group-1 peptides towards EL1

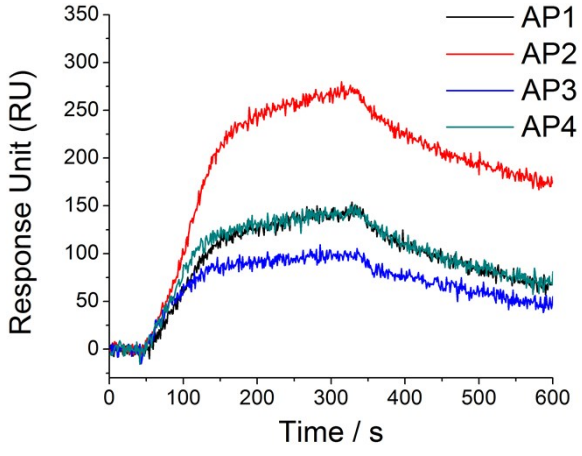


Figure S4. Sensing the peptide interactions between EL1 and group 1 peptides using SPR. SPR condition: phosphate buffer solution (10 mM PB + 50 mM NaCl, pH 6.5), [EL1-TPE] = 5 μg/mL.

5. Photostability of EL1-TPE and the binding kinetics

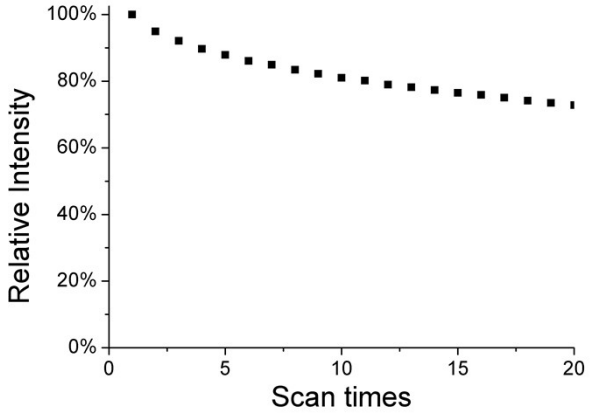


Figure S5. Photostability of EL1-TPE by continuously scanning with excitation light at 330 nm for 20 times.

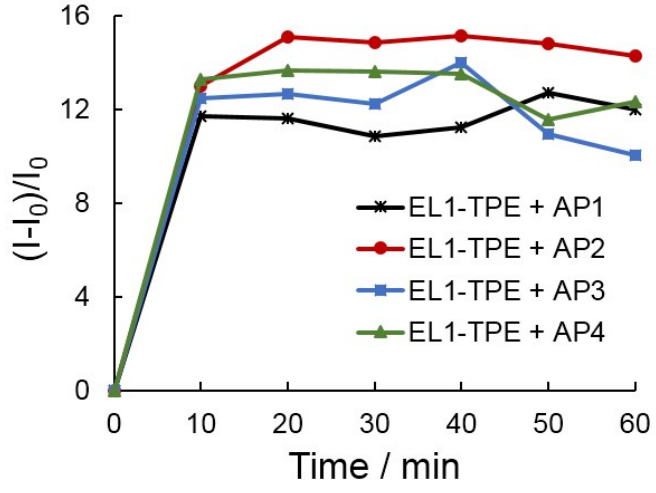


Figure S6. Binding kinetics of AP1-AP4 towards EL1-TPE in PBS of pH 7.4. *I* and I_0 represent the fluorescence intensities of EL1-TPE with and without the addition of antisense peptides respectively. λ_{ex} = 330 nm, λ_{em} = 470 nm.

6. SPR sensing the interactions between EL1 and AP5-AP8

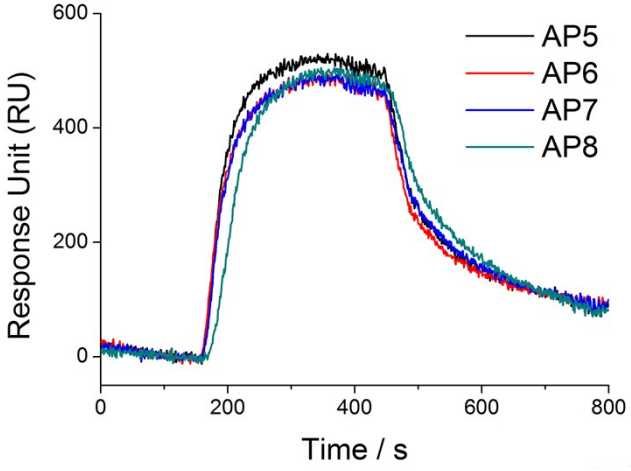


Figure S7. SPR sensing of the interactions between group 2 peptides AP5-AP8 and EL1. SPR condition: phosphate buffered saline solution (10mM PB+50mM NaCl, pH 7.0), [EL1-TPE] = 20 μg/mL.

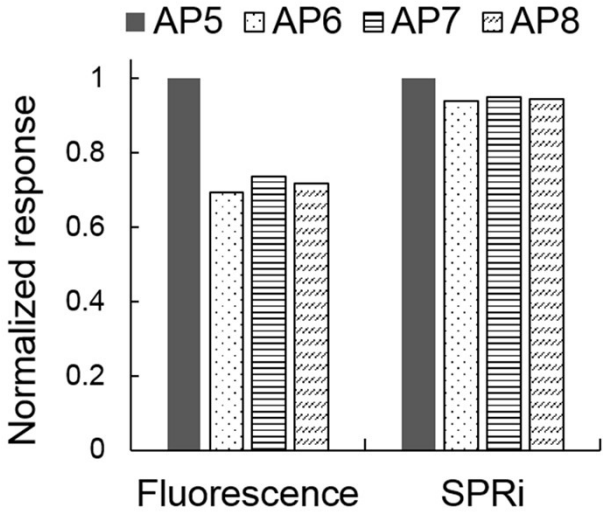


Figure S8. Comparison of fluorescence and SPR responses induced by the interactions between EL1 and different peptides. SPR conditions: phosphate buffer solution (10 mM PB + 50 mM NaCl, pH 7.0), [EL1-TPE] = 20 μg/mL.

7. Selectivity and sensitivity

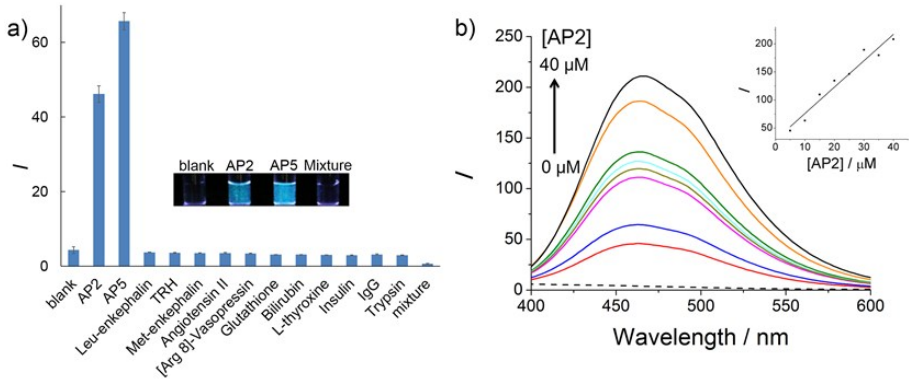


Figure S9. (a) Fluorescence signal induced by the addition of different compounds to EL1-TPE. Insert: photograph of EL1-TPE solutions under UV light (365 nm). From left: EL1-TPE solution only, EL1-TPE solution with AP2, EL1-TPE solution with AP5, and EL1-TPE solution added with the mixture containing Leu-enkephalin, thyrotropin releasing hormone (TRH), Met-enkephalin, angiotensin II, [Arg 8]-vasopressin, glutathione, bilirubin, L-thyroxine, insulin, IgG and trypsin. (b) Fluorescence spectra of the mixed solutions of EL1-TPE (10 μM) and AP2 at varied concentrations (0, 5, 10, 15, 20, 25, 30, 35 and 40 μM). Inset: linear fitting of the fluorescence intensity versus the concentration of AP2.

8. Affinity evaluation with microplate assay

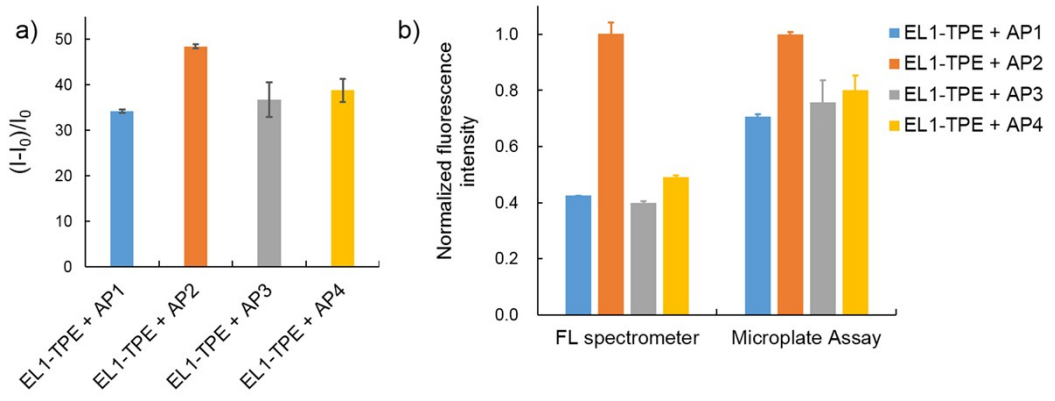


Figure S10. (a) Relative fluorescence intensities of the mixed solutions of EL1-TPE and antisense peptides respectively determined with microplate assays. I and I_0 are the fluorescence intensities of EL1-TPE with and without the addition of antisense peptides. (b) Comparison of the normalized fluorescence intensities of the mixed solutions of EL1-TPE and antisense peptides recorded with fluorescence spectrometer and microplate assays. Buffer solution: phosphate buffer solution of pH 6.5. λ_{ex} = 330 nm, λ_{em} = 470 nm.

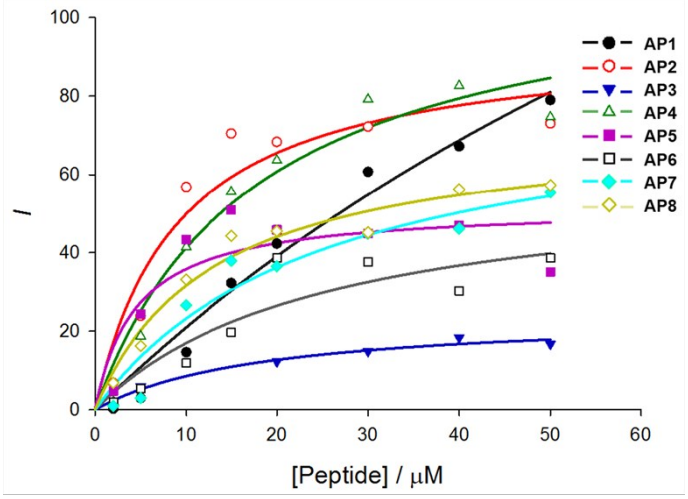


Figure S11. Fitting and quantitative calculation of binding affinity (K_D) of AP1-AP8 towards EL1 based on fluorescence measurement. The binding curves were fitted with SigmaPlot.

Table S2. The binding affinity determined by microplate assay.

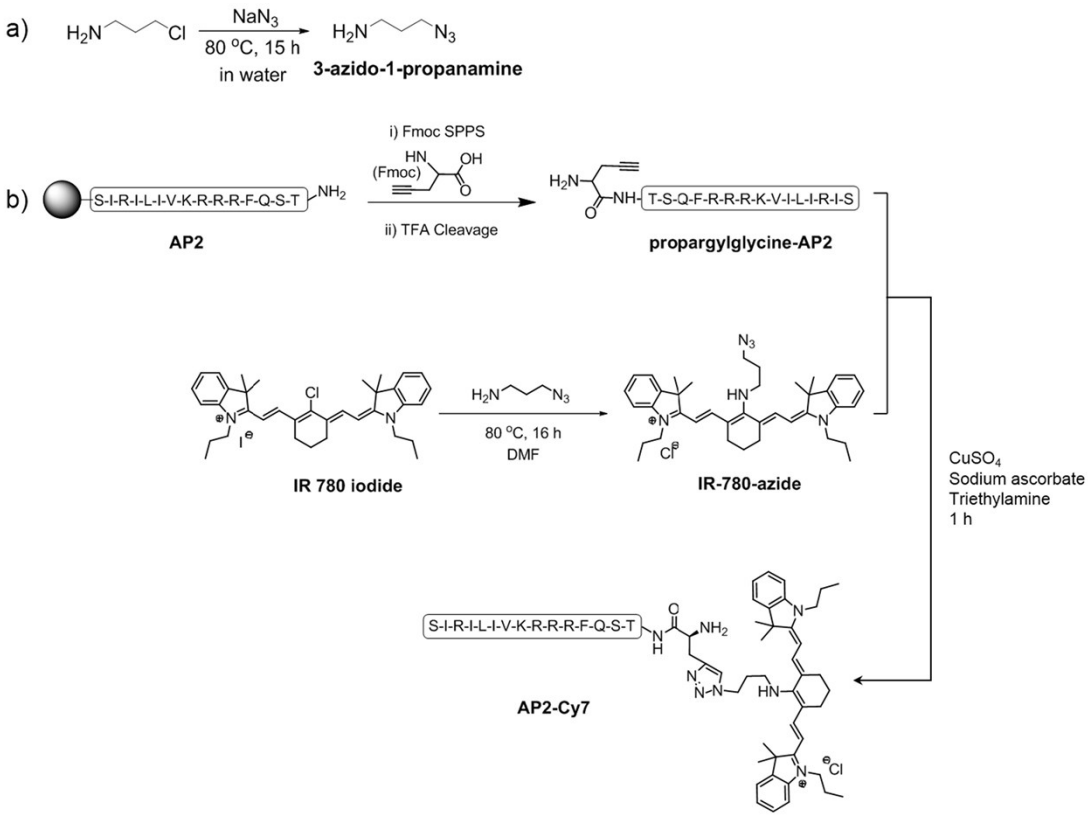
Peptides	AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8
K_D (μM)	126	9.09	18.6	17.1	4.50	25.2	25.4	12.9

9. Synthesis and characterization of Cy7-labeled peptide probes

Synthesis of 3-azido-1-propanamine. 3-chloropropylamine hydrochloride (5.0 g, 38.5 mmol) and sodium azide (8.0 g, 123.1 mmol) was dissolved in water (25 mL). The mixture was heated at 80 °C with stirring for 15 hours (Scheme S2a). After removing about 70 percent of the water by rotary evaporator, the resulting mixture was cooled in an ice bath. Diethyl ether (60 mL) and KOH pellets (10 g) were added keeping the temperature below 10 °C. After separation of the organic layer, the aqueous phase was extracted with diethyl ether (2×60 mL). The combined organic layers were dried over K_2CO_3 and concentrated. Finally, 3.2 g of light yellow oil product (3-azido-1-propanamine) was obtained, which was used directly for the next step without further purification.

Synthesis of IR-780-azide. IR-780 iodide (100 mg, 0.15 mmol) and 3-azido-1-propanamine (18 mg, 0.18 mmol) were dissolved in DMF (15 mL). The solution was heated at 80 °C for 16 hours (Scheme S2b). The solvent was removed under vacuum and the residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH (v/v, 20:1), obtaining IR-780-azide as a deep blue solid (47 mg, 52%).

Synthesis of Cy7-labeled peptides. The Cy7 labeling was realized by click reaction (Scheme S2b). Before that, peptides were modified with propargylglycine via on-resin conjugation by standard FMOC SPPS. After purification, 1 equiv of propargylglycine-modified peptides (0.29 mM), 1.2 equiv of $CuSO_4$ (0.35 mM) and 1.2 equiv of IR-780-azide (0.35 mM) were mixed in solvent (H_2O:DMF=1:2, v:v) containing 1% (v/v) trimethylamine. With N_2 gas protection, 2.4 equiv sodium ascorbate (0.70 mM) was added. After being stirred in the dark for 1 hour at room temperature, the reaction mixture was analyzed and purified with HPLC. The products AP2-Cy7 and AP5-Cy7 were obtained as blue powders. They were characterized with MS (ESI, positive, AP2-Cy7 m/z $[(M+2H)/2]^{2+}$ calcd: 1286.57; found: 1285.74; AP5-Cy7 m/z $[(M+2H)/2]^{2+}$ calcd: 1209.50, found: 1208.85). The purities were verified with HPLC on a Diamonsil C18(2) column (250 × 4.6 mm).



Scheme S2. Synthetic route for AP2-Cy7.

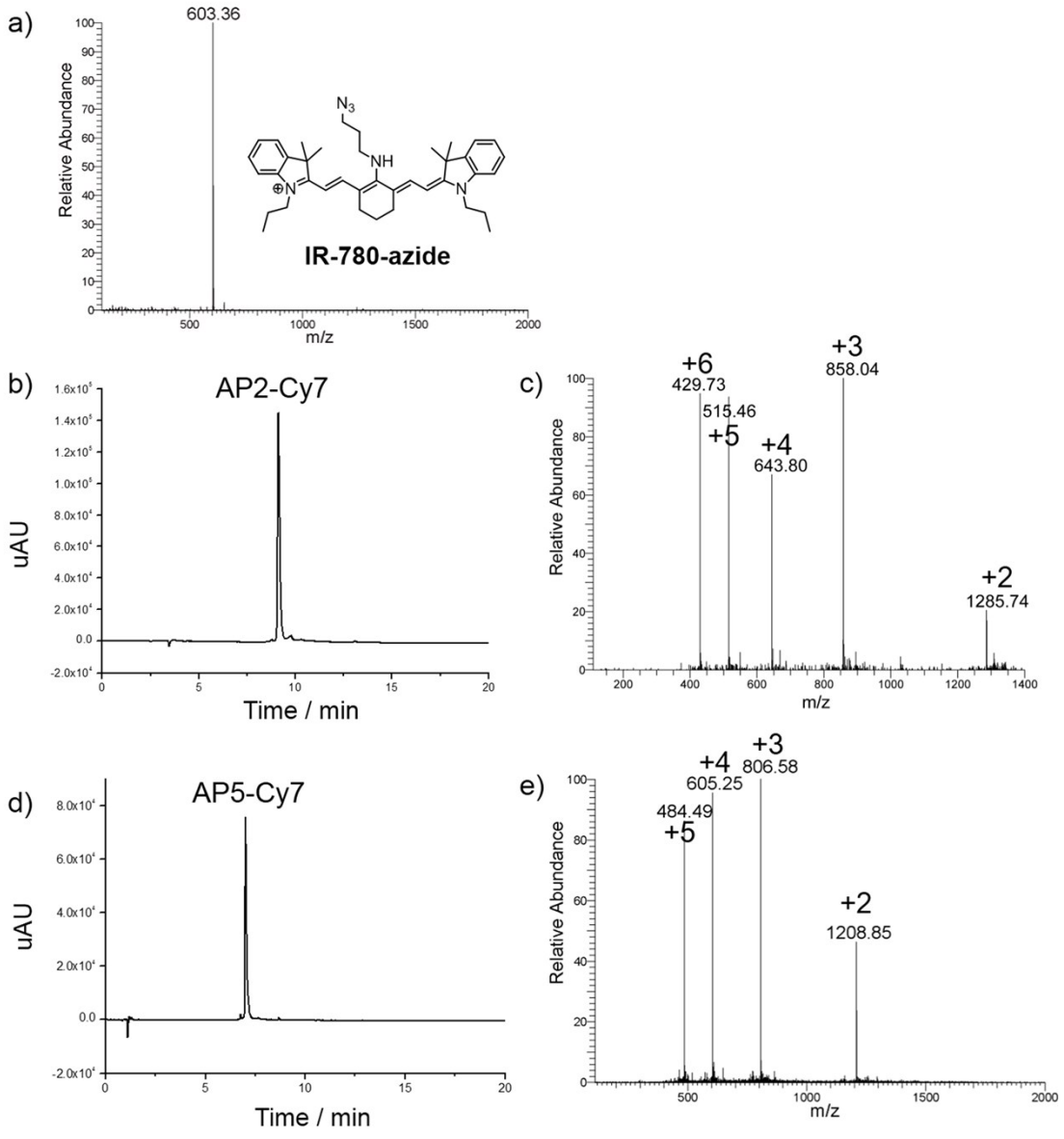


Figure S12. (a) ESI-MS characterization of **IR-780-azide** (ESI positive, m/z $(M+H)^+$, calcd:603.42; found: 603.36). (b-e) HPLC analysis and MS characterization of Cy7-labeled peptide probes. (b) HPLC analysis of AP2-Cy7. (c) ESI-MS characterization of AP2-Cy7 (ESI positive, m/z $[(M+2H)/2]^{2+}$, calcd: 1286.57; found: 1285.74). (d) HPLC analysis of AP5-Cy7. (e) ESI-MS characterization of AP5-Cy7 (ESI positive, m/z $[(M+2H)/2]^{2+}$, calcd: 1209.50; found: 1208.85). HPLC separation was performed on a Diamonsil C18(2) column (250 × 4.6 mm) monitored at the wavelength of 650 nm. Gradient: 0-20-20.01-24min, 10%B-80%B-100%B-100%B (A: H_2O containing 0.1% fomic acid, B: acetonitrile containing 0.1% fomic acid).

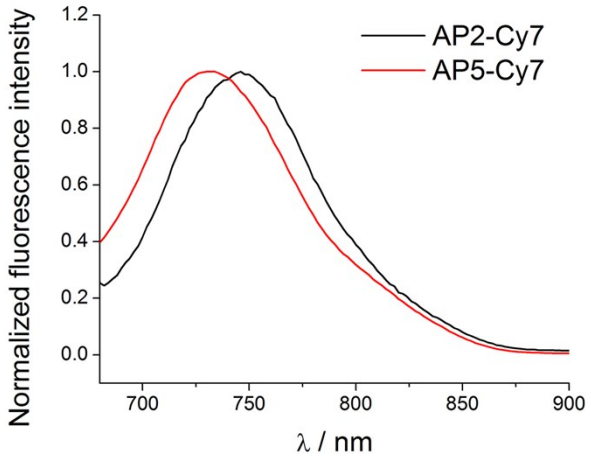


Figure S13. Fluorescence spectra of AP2-Cy7 and AP5-Cy7. λ_{ex} = 650 nm.

10. Specific imaging of cancer cells

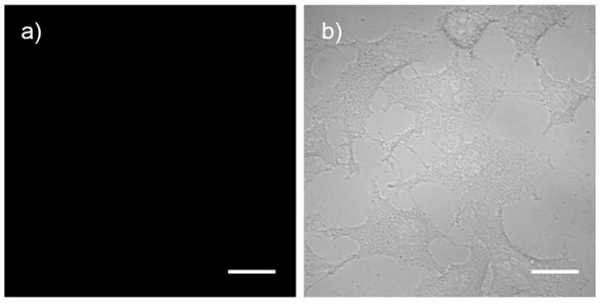


Figure S14. Auto-fluorescence from HepG2 cells. (a) Fluorescence image under the same condition as used for AP2-Cy7 treated cells; (b) bright field image. Scale bar: 20 μm.

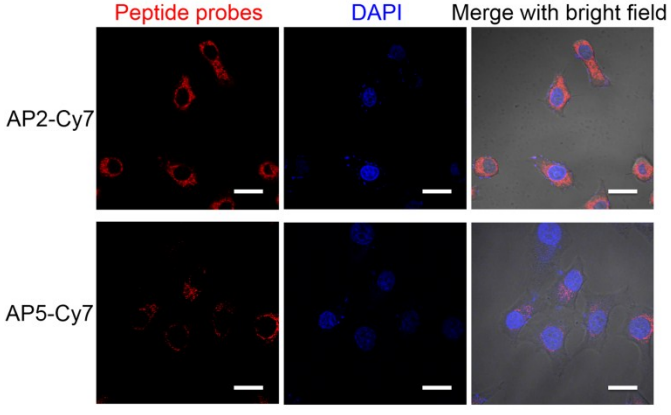


Figure S15. Co-staining of HepG2 cells with peptide probes (AP2-Cy7 and AP5-Cy7, respectively) and nucleus indicator DAPI. Scale bar: 20 μm.

11. Cytotoxicity assay of the screened peptides

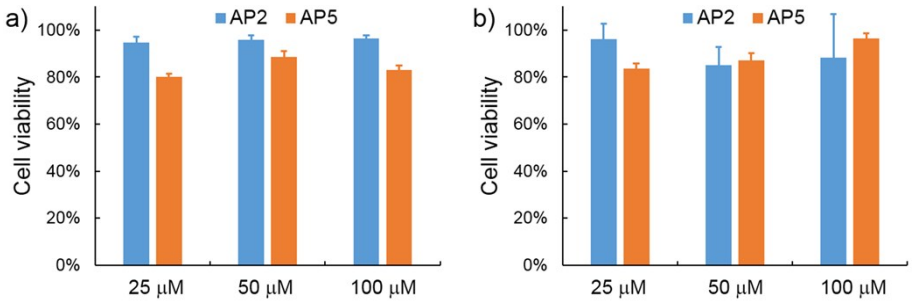


Figure S16. Cytotoxicity assay of AP2 and AP5 based on MTT assays. (a) Cell viability of HepG2 cells after 48-hourtreatment. (b) Cell viability of HEK293 cells after 48-hourtreatment.