Electronic Supplementary Information

Switchable Probes: pH-Triggered and VEGFR2 Targeted Peptides Screening through Imprinting Microarray

Yixia Qian[†a,b,c], Weizhi Wang[†§*a], Zihua Wang[a], Qiuju Han[a,d], Xiangqian Jia[a,d], Shu Yang[a] and Zhiyuan Hu*[a]

Contents

1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, Wang resin, and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) were purchased from GL Biochem (China). Tentagel Resin was from Rapp Polymere (Germany, loading 0.35mmol/g). Trifluoroacetic acid (TFA), thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), fluorescein isothiocyanate (FITC), hoechst 33342, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) and streptavidin coated magnetic beads (1 μm) were from Sigma-Aldrich (USA). N-methyl morpholine (NMM), piperidine and N, N'-dimethylformamide (DMF) were all from Beijing Chemical Plant (China). 1,2-Ethanedithiol (EDT) was from Alfa Aesar (USA). Triisopropylsilane (Tips) was from Acros Organics (USA). Cyanogen bromide (CNBr) was from J&K Chemica (China). Silicon wafer (N/1-0-0, 500 μm) was from KYKY Tech (China) and the gold surface SPRi chip was Plexera Nanocapture bare gold chip with a gold layer of 47.5 nm thickness and size of 25 mm×75 mm. VEGFR2 protein was from Sino Biological Inc (Beijing, China). Biotin labeling kit was from SoluLink. PE anti-human VEGFR2 was from Biolegend (CA, USA). FITC-tagged CD31 antibody was from Abcam (UK). DMEM/high glu-cose medium, RPMI-1640 medium and trypsin were pur-chased from GE Healthcare Life Sciences. The human umbilical vein endothelial cell line HUVEC, human embryonic kidney cell line 293T , human ovarian cancer cell line SKOV3 and human colonic adenocarcinoma cells HT-29 were purchased from Cell Resource Center, Chinese Acad-emy of Medical Sciences (China). All cell lines were sup-plemented with 10% fetal bovine serum (FBS) (Gibco) and 100 U/mL penicillin and 100 U/mL streptomycin (Gibco).

2. Fabrication processes of bi-functional microchip

To fabrication the micro-well array chip, a 4-inch (100) silicon wafer was employed with 500 μm in thickness which fulfilled the requirement of Bruker ULTRAFLEXTREME mass spectrometer. Using a sputtered aluminum layer (200 μm thick) as mask (Figure S1a), micro-wells were formed by ICP (induced couple plasma) etching. The geometric parameters of micro-wells were 230 μm ×230 μm ×200 μm, and size of micro-well could be varied by redesigning the photo-mask of lithography to fit the sizes of different micro-beads. After removing the aluminum layer by wet etching, a titanium layer (30 nm) and a silver layer (200 nm) were sequentially coated on the silicon wafer. Titanium was served as adhesive materials. Silver was used because it had high conductivity for MS detection and good chemical stability. Figure S1b showed the fabrication process.

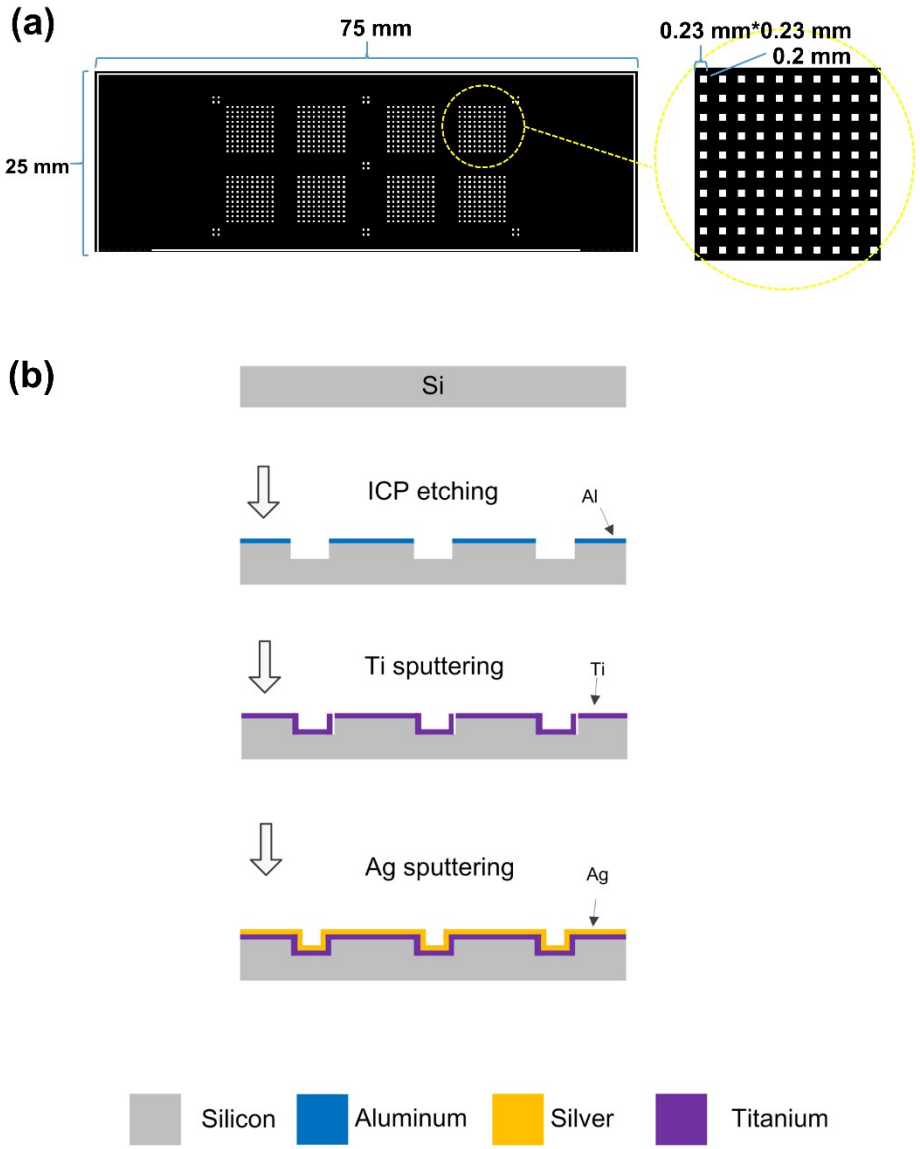

Figure S1. The mask of the microarray chip (a) and the fabrication process of the chip (b).

3. Construction and synthesis process of the OBOC peptide library towards VEGFR2

In our strategy, a 15-mer OBOC combinatorial library was constructed. As the major vascular endothelial growth factor (VEGF) receptor, VEGFR2 had high affinity towards the vascular endothelial growth factor-A (VEGF-A) and vascular endothelial growth factor-C (VEGF-C).[1] We designed the peptide library referenced from the sequences of VEGF-C 113-155 amino acid segment. Additionally, Methionine was used for assisting in situ chemical cleavage. Glycine was used as an extend linker to reduce the steric hindrance. Cysteine was used to provide the thiol group in the following SPRi detection. The detailed synthesis process of the OBOC peptide library was described in Figure S2. So the peptide library consisted of the alterable sequences (X) and the fixed sequences. The general sequence was $CX_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}$ $X_{13}X_{14}X_{15}$G M, in which X_3, X_{13}, X_{14}, represented the conservative amino acids Aspartic acid (D), Leucine (L), Serine(S), respectively. They were reported to improve the interaction with VEGFR2 protein.[1] In order to make a diversity peptide library, the amino acid at position of X_1, X_2, $X_{4\text{-}12}$, X_{15} were designed with acidic, basic, polar, or nonpolar residues. As the sequence on each bead were randomly distributed in the OBOC library, the capacity was $4\times5\times1\times5\times5\times2\times3\times5\times2\times3\times3\times$ $2\times1\times1\times2=1080000$. We set the five times library redundancy. Therefore, we carried out the high throughput peptides screening from the 5.4×10^6 candidates.

The OBOC library was synthesized by employing the Fmoc (9-Fluorenylmethoxycarbonyloxy) strategy SPPS (solid phase peptide synthesis). Tentagel Resin (loading 0. 35 mmol/g) was used as the solid phase support. Figure S2 showed the synthesis process. All the synthesis process was carried out in dehydrous DMF. During the coupling step, the HBTU (4 mmol) Fmoc-amino acid (4 mmol) reagent was dissolved in 0.4 mol/L NMM (4 mL) in DMF and the coupling time was

40 min. In the deprotection step, 20% (v%) piperidine was used to remove the Fmoc group and the deprotection time was 10 min. During the OBOC library synthesis, solid support beads were split equally in each cycle and different amino acids were added. It means that amino acid coupling process was carried out in the "split" step while the deprotection process was carried out in "pool" step. After elongation, cleavage reagent (92.5% (v%) TFA, 2.5% (v%) water,2.5% (v%) EDT and 2.5% (v%) Tips) was introduced into the vessel to cleave the SP (side chain protecting group) of each residue (2 hrs). All the above experiments were carried out in the solid phase peptide synthesis vessels with sieves in it. For the *in situ* chemical cleavage in the microwells, 30mg/mL cyanogen bromide (BrCN) solution was used overnight. In the follow Figure S2, NMM is the abbreviation for N-Methyl morpholine, DMF is the abbreviation for N, N-dimethylformamide, TFA is the abbreviation for Trifluoroacetic acid and SP is stand for side chain protecting group.

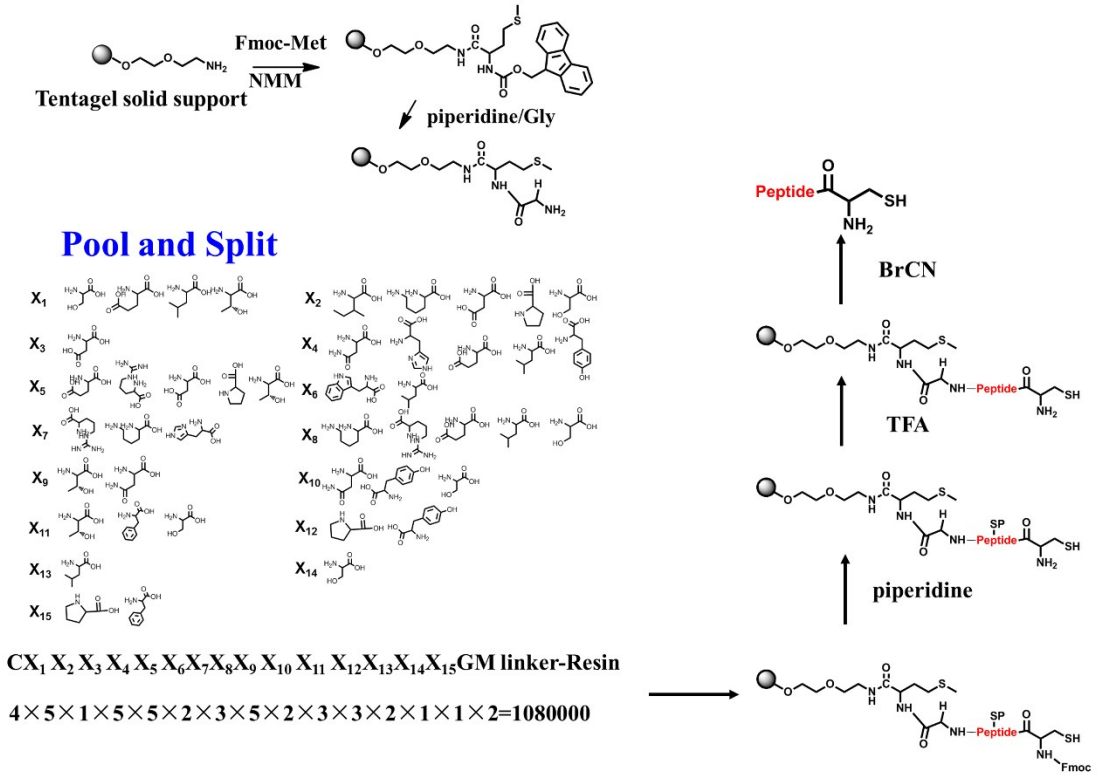

Figure S2. Synthesis process of the OBOC peptide library towards VEGFR2.

4. The biotinylation process of the VEGFR2 protein

The VEGFR2 was biotinylated using the biotinylation kit (SolulinkTM, ChromaLink Biotin Catalog# B-9007-105K)(figure S3). The labelling process was based on an advanced protein biotinylation reagent containing an aromatic, N-hydroxy-succinimidyl ester functional group (a), which efficiently modified protein lysine residues in a phosphate buffer system. The linker possesses an embedded bis-arylhydrazone structure (b), which formed the compound's UV-traceable chromophore. The traceable nature of this chromophore enabled the non-destructive quantification of biotin (c), attached to the protein. These three chemical elements (a, b and c) were linked together through a long-chain PEG3 spacer (d), which preserved SA/biotin affinity while simultaneously maintaining protein solubility. Using this kit, the protein VEGFR2 was biotinylated with an average of 2.4 biotins per molecule.

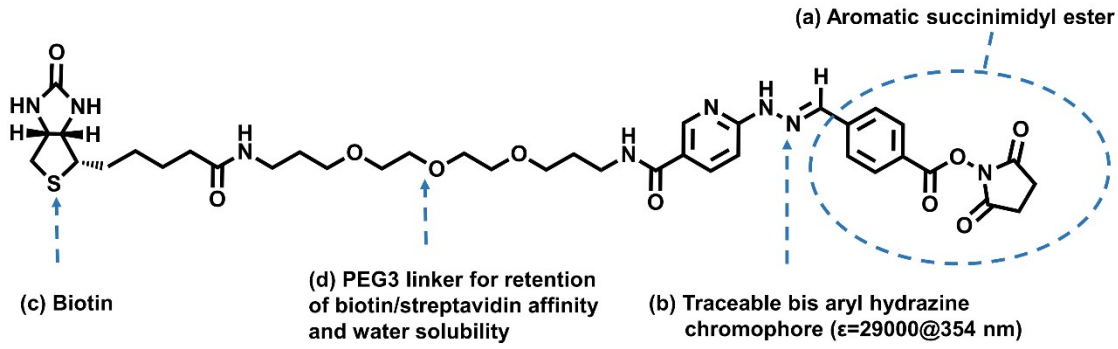

Figure S3. Structure and function of ChromaLink Biotin.

5. Positive peptide trapping by magnetic field through the microchannel

Schools of peptide beads were incubated with biotinylated VEGFR2 and streptavidin coated immunomagnetic beads successively. Then peptide beads were introduced into a Teflon tube (diameter: 1 mm, flow rate 600 μL/min) with a magnet closely next to the outer wall of the tube. Finally, magnet was removed and trapped beads were flushed out and collected. The mixture of positive peptide beads and streptavidin coated magnetic beads were suspended in a tube, and centrifuged for a very short time to collect the beads at the bottom. The process was shown in Figure S4.

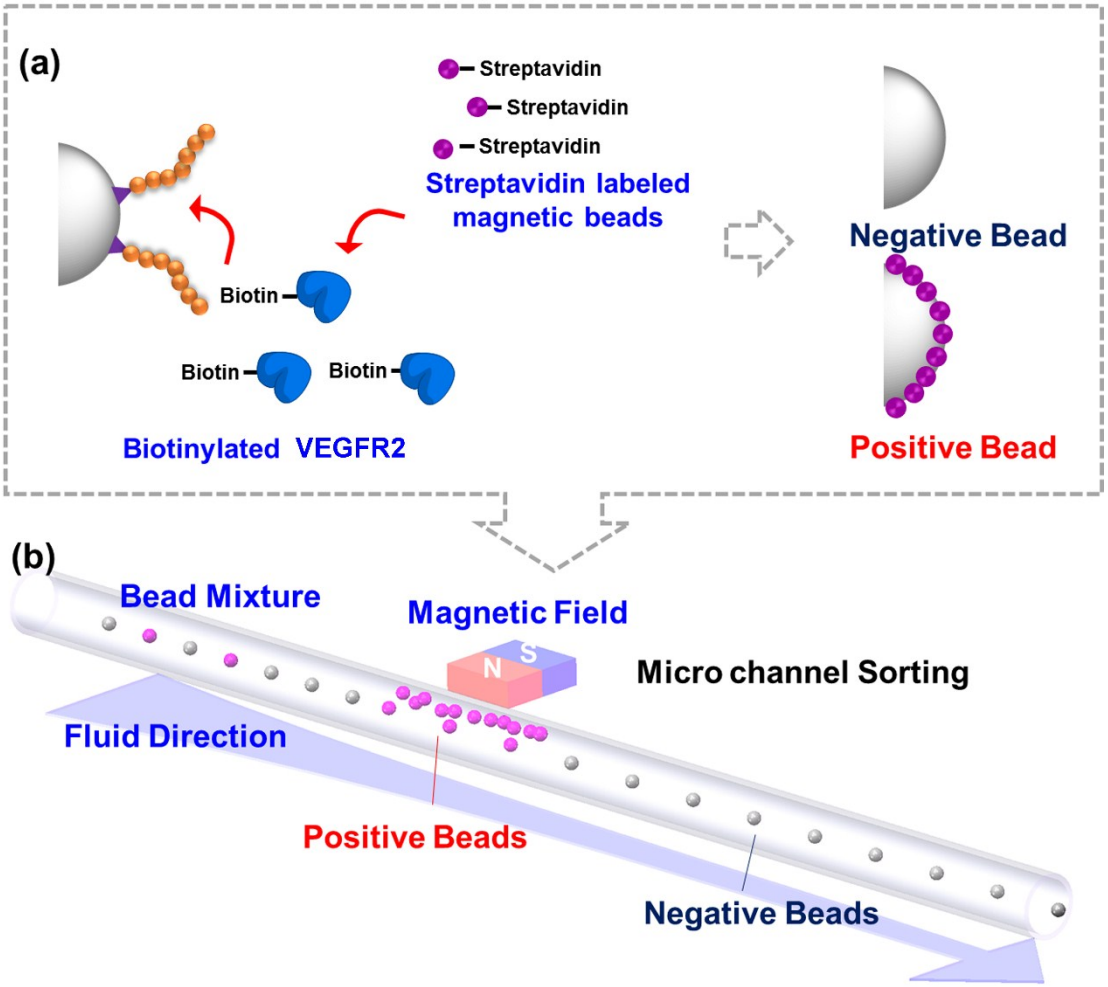

Figure S4. Positive peptide trapping by magnetic field through the microchannel.

6. Chip imprinting and SPRi detection of the microarray

CHCA (α-cyano-4-hydroxycinnamic acid) was dissolved in Solvent TA30 (30:70 [v/v] Acetonitrile: TFA 0.1% in water)/CNBr (1/1) to prepare a matrix and cleavage solution. Peptide beads was suspended in matrix solution and loaded into the wells of both the two units. One gold-coated chip was pressed on one unit with the gold surface down onto the microarray chip and the other one was pressed on the other unit correspondingly. The chip was incubated in 4^{o}C overnight in water box. The gold coated chip was blocked by 5% (m/v) non-fat milk. The SPRi analysis procedure follows the following cycle of injections: running buffer (PBST, baseline stabilization); sample (five concentration of the protein, binding); running buffer (PBST, washing); and 0.5% (vol/vol) H_3PO_4 in deionized water (regeneration). Protein VEGFR2 were diluted into 10 μg/mL with PBST and multiple proportion diluted into 5 μg/mL, 2.5 μg/mL, 1.25 μg/mL and 0.625 μg/mL. Real-time binding signal were recorded and analysed by SPRi system. The PlexArray® Analyzer was used to process the data. The silver chip and the gold chip was shown in Figure S5a and the imprinting microarray on the gold chip was shown in Figure S5b. The detail structures of the microarray chip were shown in Figure S5c-e.

Among the 400 spots, 24 spots showed relative low dissociation constant during the acidic environment detection and 17 ones during the neutral environment detection. These positive spots were highlight and marked in both detection conditions. Since the peptide beads in the microwell array chip were addressed bead-to-spot with the SPRi chip, the beads in the corresponding wells with the highlighted spots were characterized through the TOF-TOF MS detection. The spectrum of each well was characterized by uploading the data to MASCOT database (Figure S6). Finally, the positive peptide sequences in both experimental conditions were aligned by using the software Clus-talX2. When in the acidic environment, the conserved sequences emerged that the motif was XX-Asp-Glu-Glu-Trp-XXXXX-Pro-Leu-Ser-X (Figure S6a). For the neutral environment, the conserved sequences followed the motif XX-

Asp-His-Glu-Trp-XXXXX-Pro-Leu-Ser-X (Figure S6c). The consensus sequences that emerged with the most frequent matching among all fifteen residues were SKDEEWHKNNFPLSP (STP) for acidic environment and TIDHEWKKTSFPLSF (TP) for neutral environment. The dissociation constants were calculated from dynamic constants obtained by curve-fitting of the association and dissociation rates to real-time binding and washing data. For STP, the KD values were calculated as 8.50×10^{-8} mol/L in the acidic condition (pH 5.8) and 4.52×10^{-5} mol/ Lin the neutral condition (pH 7.4), respectively (Figure S6b), which showed a high binding affinity in the acidic environment. Meanwhile，STP had really low binding affinity in the neutral environment. For TP, the corresponding KD values were 5.99×10^{-6} mol/L and 5.93×10^{-7} mol/L (Figure S6d). All these SPRi results were obtained from two parallel tests and adopted the average values.

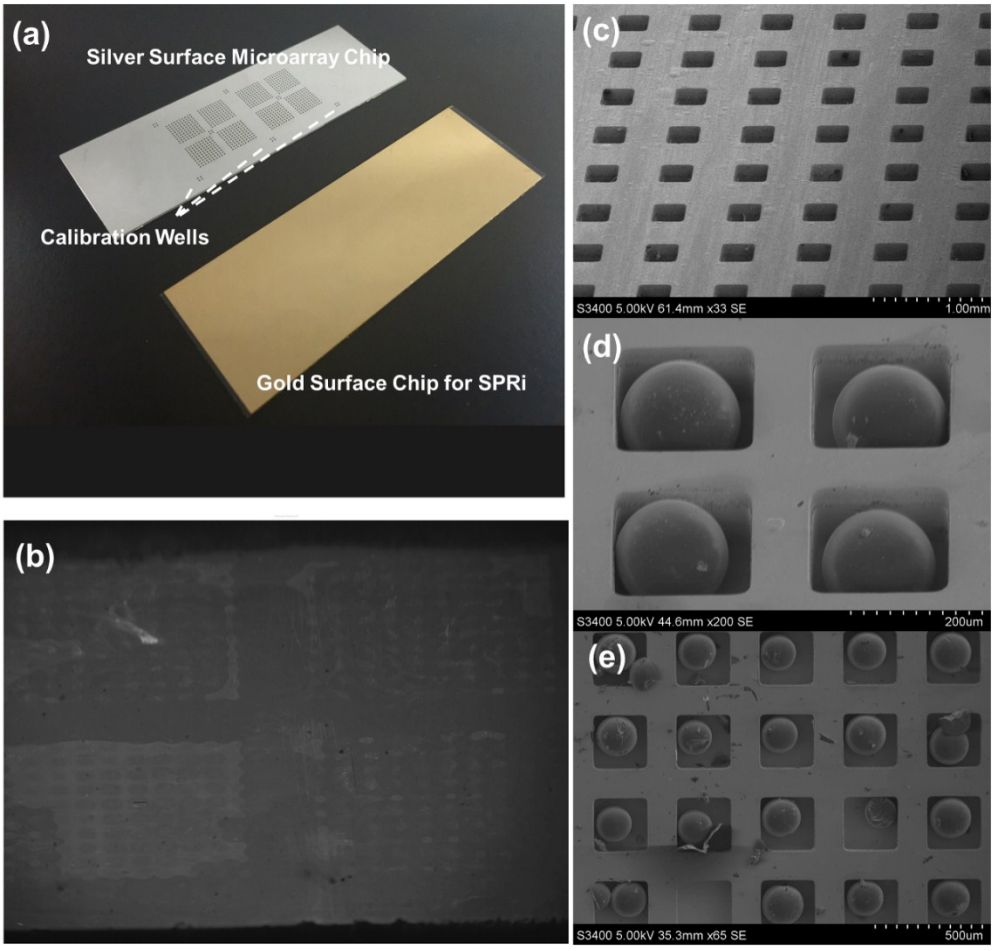

Figure S5. (a) The photograph of the silver chip and the gold chip. (b) The imprinting microarray on the gold chip. (c-e) The detail structures of the microarray chip and the

trapping of the peptide beads.

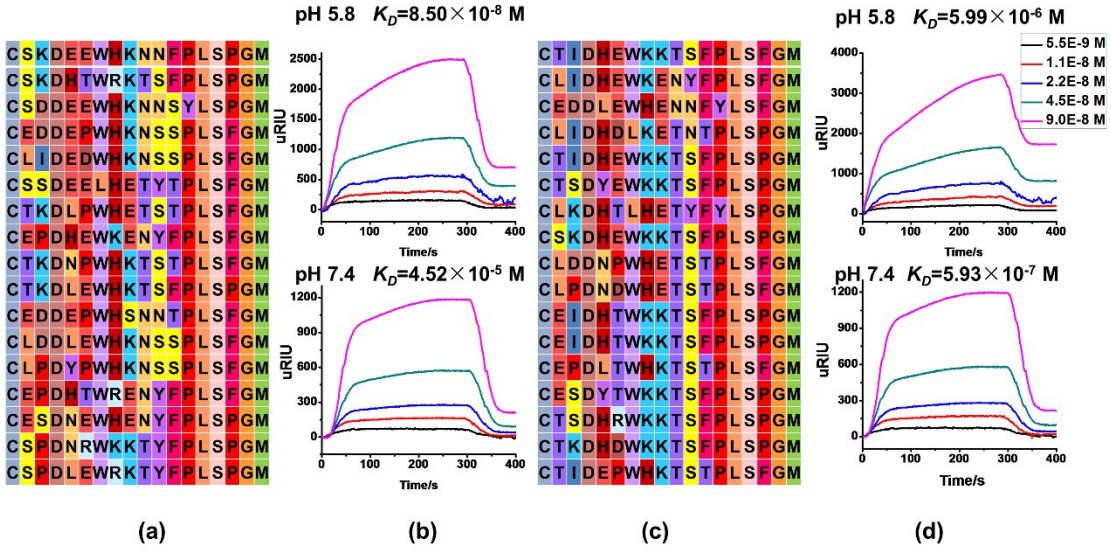

Figure S6. Alignment of peptides using the ClustalX2 multiple alignment tool.(a) in the acidic environment, (c) in the neutral environment, and SPRi detection of the binding affinity of STP (b) and TP (d) toward VEGFR2 at pH 5.8 and 7.4,respectively.

7. *In situ* MALDI-TOF sequencing and identification of peptides

After cleaving the positive peptide beads *in situ*, MALDI-TOF MS analysis was performed on a Bruker ULTRAFLEXTREME mass spectrometer (Bruker Daltonics, Germany) equipped with a nitrogen laser with reflectron and positive-ion modes. The laser power energy was adjusted between 0% and 100% to provide laser pulse energy between 0 and 100 μJ per pulse. The mass spectra were typically recorded at an accelerating voltage of 19 kV, a reflection voltage of 20 kV, and with laser pulse energy of 60 μJ. Each mass spectrum was acquired as an average of 500 laser shots. For *in situ* "one well one bead" analysis, the characteristic TOF-TOF MS spectra of the peptides screened out from the high throughput library was shown in Figure S7. Figure S7a showed the representative TOF-TOF MS spectrum at pH 5.8. Figure S7b showed the representative TOF-TOF MS spectrum at pH 7.4.

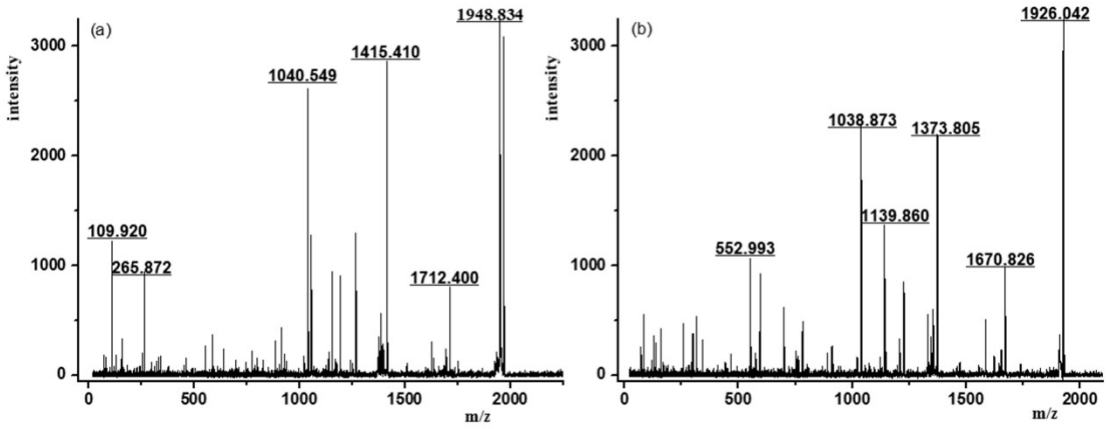

Figure S7. Representative TOF-TOF MS spectrum for the positive peptide beads cleaved off from individual beads. (a) Representative TOF-TOF MS spectrum obtained at pH 5.8. (b) Representative TOF-TOF MS spectrum obtained at pH 7.4.

8. *In situ* MALDI-TOF sequencing and identification of peptides *de nove*

When the positive sequences were determined，the peptides were synthesized *de novo*. Peptides were synthesized through the Fmoc strategy. Here the FITC-STP and FITC-TP were resynthesized for the *in vitro* confocal fluorescence imaging experiments of the peptides binding toward living cells. STP and TP with a cysteine labelled at each N terminal (C-STP and C-TP) were used for SPRi detection to confirm the affinity. Wang Resin was used as the solid phase support. All the synthesis process was carried out in dehydrous DMF in the solid phase peptide synthesis vessels with sieves in it. In the coupling step, the Fmoc-amino acid reagent was dissolved in 0.4 mol/L NMM in DMF and the coupling time was 40 min. In the deprotection step, 20% (v%) piperidine was used to remove the Fmoc group and the deprotection time was 10 min. The FITC labelled peptide was linked with an aminocaproic acid before they were linked to FITC. During this synthesized process, the solid phase peptide synthesis vessels was covered with tin foil paper. After elongation, cleavage reagent (95% (v%) TFA, 2.5% (v%) water,2.5% (v%) EDT) was introduced into the vessel to cleave the resins and SP (side chain protecting group) of each residue (2 hrs). For the peptides with a cysteine labelled at the N terminal, the cleavage reagent was composed of 92.5% (v%) TFA, 2.5% (v%) water,2.5% (v%) EDT and 2.5% (v%) Tips. The crude peptides were analyzed and purified by using a Waters HPLC (High Performance Liquid Chromatography) system (Waters e2695) on a SunFire® C18 column (5 μm，4.6×250 mm) at a flow rate of 1.0 Ml/min. Gradient: 0-25 min, 5-70% acetonitrile containing 0.1% TFA. Figure S8 showed the purified peptide and the structure of FITC-STP (a), C-STP (b), FITC-TP(c) and C-TP(d). The purified peptides were characterized by UPLC (Ultra Performance Liquid Chromatography)-MS system (Waters Acquity UPLC® H-class). Figure S9 showed the mass spectrum of FITC-STP (a), C-STP (b), FITC-TP(c) and C-TP(d).

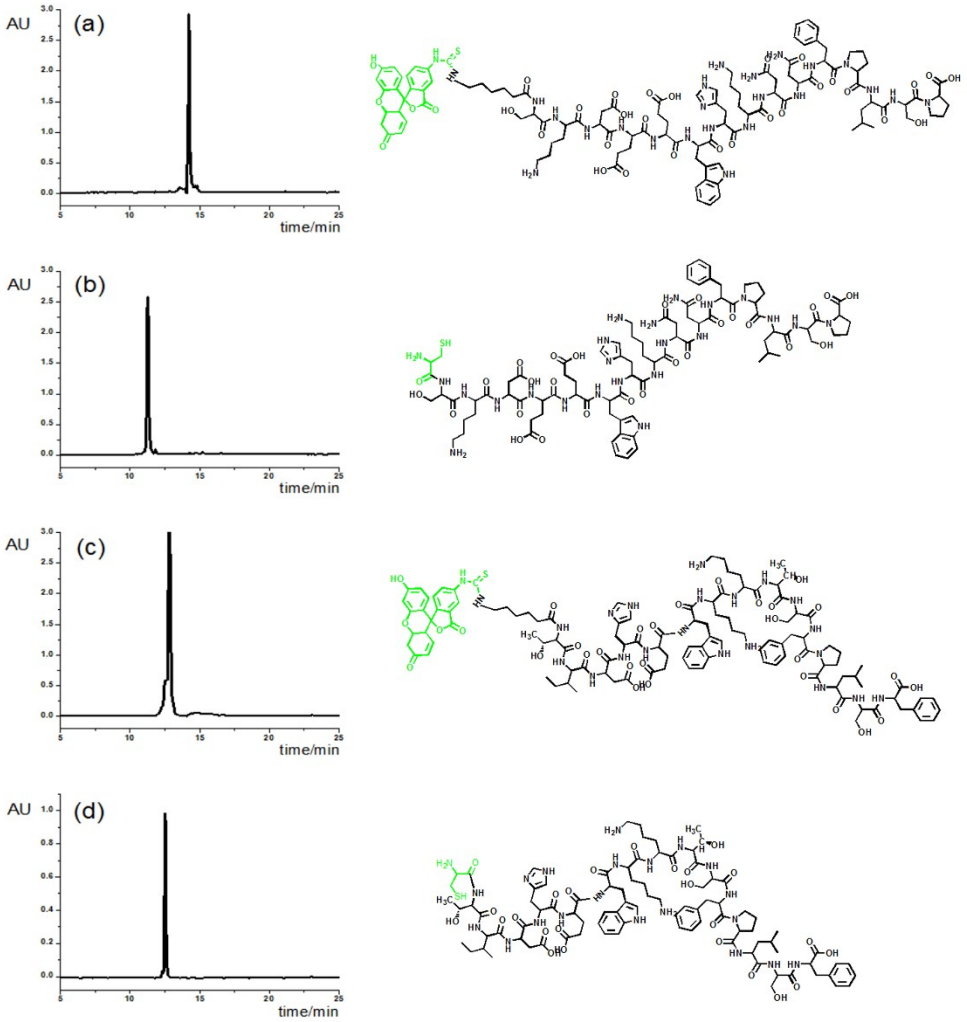

Figure S8. Characterizations of HPLC and structures for FITC-STP (a), C-STP (b), FITC-TP (c) and C-TP (d).

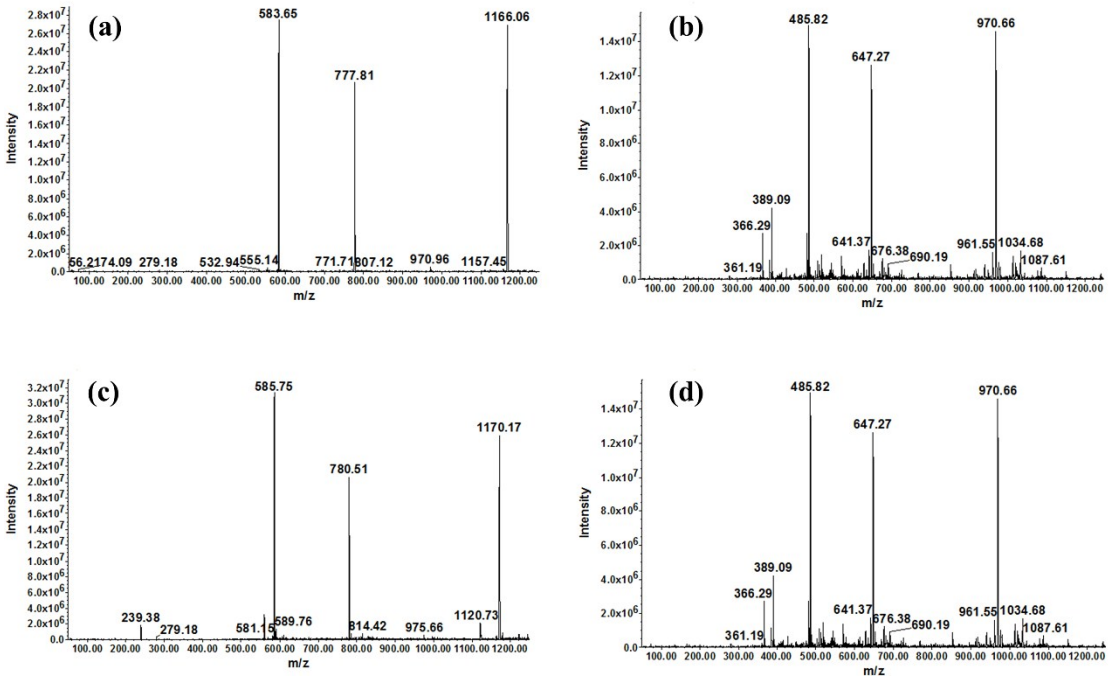

Figure S9. Characterizations of mass spectrum for FITC-STP, C-STP, FITC-TP and C-TP.

9. Binding affinity confirmation of the peptides towards negative cells

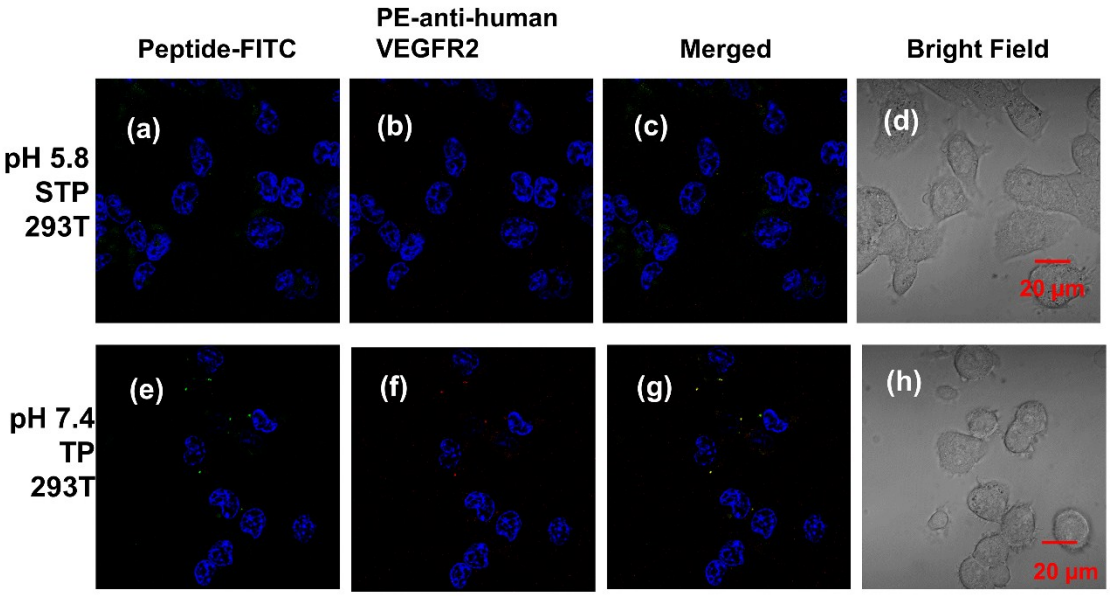

Figure S10. FITC-STP interacted with 293T with PE-anti-human VEGFR2 at pH 5.8 (a-d). FITC-TP interacted with 293T with PE-anti-human VEGFR2 at pH7.4 (e-h).

10. MTT assay of the peptides towards HUVEC, SKOV3 and 293T cells

MTT assay was used to measure the cell growth or inhibition by the peptide. HUVEC cells with high expression of VEGFR2 proteins, SKOV3 cells with low expression of VEGFR2 proteins and 293T cells with none VEGFR2 proteins expressed were used. 4000 of each cells were seeded overnight in each well of a 96-well plate in DMEM medium.100 μL peptides STP with 5 concentrations was added in each well for 48 hours. Removed the solution and added MTT solution (1mg/mL) to each well and plate for 4 hours at 37°C. Then the solution was removed and 150 μL dimethyl sulfoxide (DMSO) was added to each well. After 5min vibration mixing, the optical density (OD) at 570nm was measured using an ELISA reader. The survival rates of HUVEC cells, SKOV3 cells and 293T cells exposed to STP and TP at the concentration from 1 nM to 10 μM were close to 100%. These results indicated that STP and TP showed good biocompatibilities and might be safely targeting the VEGFR2 with a very low toxicity.

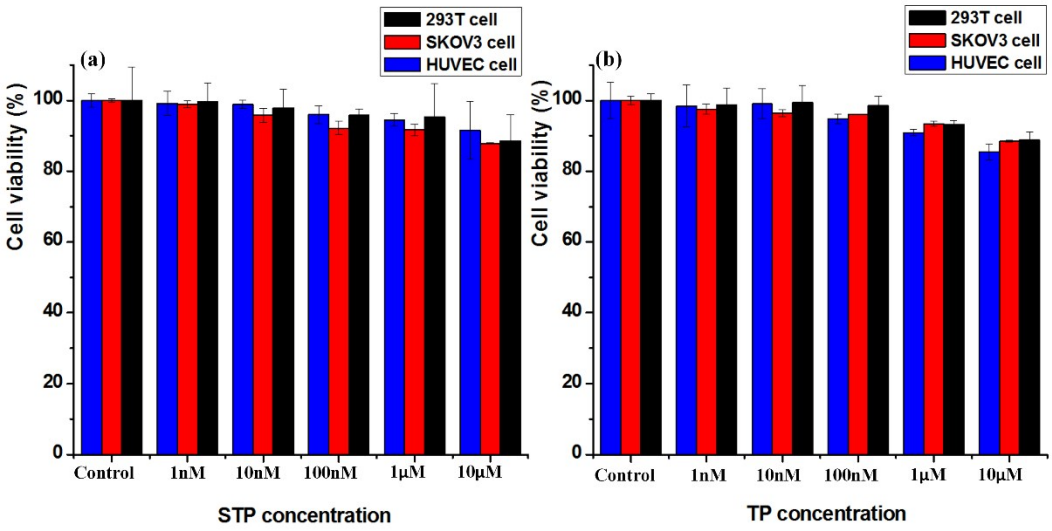

Figure S11. Activities of peptides STP (a) and TP (b) on HUVEC, SKOV3 and 293T cells growth were measured by MTT assay (Standard error of the mean were obtained from three independent experiments, n=3).

11. Tracking the binding process of the STP peptide towards HUVEC.

The initial binding of STP to the HUVEC cell membrane was demonstrated by the Z-Stack Image (Figure S12). After incubating the cells with FITC-STP at pH 5.8 for 20 min. Scanned the cross-sections of the cells along the Z-axis. The shape of the circular fluorescence changed continuously. The results showed STP not only bound to the cell membrane, but also had released into the cytoplasm at 20 min, which further confirmed STP had potential to be used as a penetrating peptide.

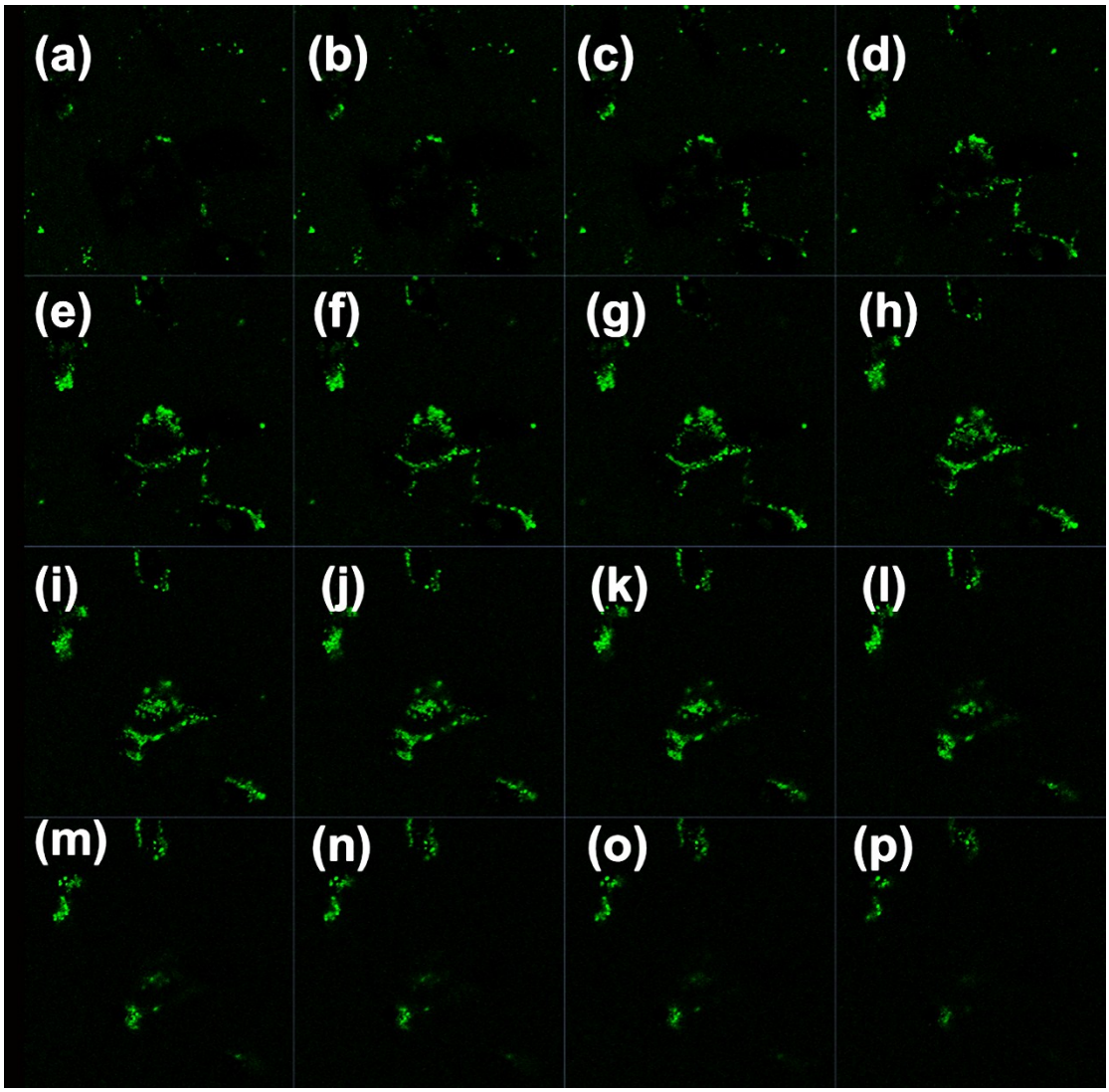

Figure S12. Z-stack images of HUVEC cells. After incubating the FITC-STP, HUVEC cells were scanned along the Z-axis (0.56 μm slide^{-1}).

12. Cell culture and *in vitro* confocal fluorescence imaging experiments of the peptides binding toward living cells.

The human umbilical vein endothelial cell line HUVEC and human embryonic kidney cell line 293T were cultured in Hyclone DMEM/high glucose with 10% FBS, human ovarian cancer cell line SKOV3 was cultured in McCoy's 5A culture medium with 10% FBS, 1% penicillin and streptomycin at 37°C containing 5% CO_2. For 293T cells, in order to increase cell adherent ability, polylysine was used to coat culture dishes before seeding the cell. After removing the polylysine, approximately 1×10^5 cells/mL were seeded into culture dishes and cultured overnight for the adhesion of cells. FITC-labeled peptide (STP or TP) was dissolved in cold DMEM (pH 5.8 or pH 7.4) at a concentration of 1 mg/mL. First, the cells were incubated with 200 μL DMEM with 0.1% v/v Hoechst 33342 in dark at 37°C for 15min.Then the cells were incubated with FITC-labeled peptide solution in dark at 4°C for 10min. For the peptide specificity experiment, 5 μL PE anti-human VEGFR2 dissolved in 200 μL DMEM was first added into cells at 4 °C in dark for 2 hours. Then removed the solution and added 200 μL DMEM with 0.1% v/v Hoechst 33342 and 1 mg/mL peptide, respectively. Finally the cells were washed three times with cold DMEN. Confocal fluorescence imaging was performed on a ZEISS LSM710 confocal laser scanning microscope. A 488 nm laser was the excitation source for FITC throughout the experiment and emission laser was at 525 nm. Hoechst 33342 was excited at 405 nm and emitted at 461 nm, PE anti-human VEGFR2 was excited at 514 nm and emitted at 578 nm. The objective lens used for imaging was a 63× oil immersion objective (ZEISS).

13. Circular dichrosim studies.

The CD spectra of the peptides were scanned by using a Jasco J-1500 CD spectromrter. All spectra were obtained in a sample chamber flushed with nitrogen. The spectra were scanned in capped quartz optical cells with 1 cm path length. Spectra were collected from 185 nm to 300 nm. STP and TP peptides were taken as a 0.33 mg/ ml stock solution in water at pH 5.8 and pH 7.4, respectively. Then they were diluted into the appropriate water to obtain a 0.02 mg/ml solution to scan the spectrum. All θ values were expressed as degrees centimetre squared per decimole. To obtain the CD spectra at varying pH, the stock peptide was diluted into water, and the pH was adjusted to the required value by using a few microliters of sodium hydroxide or hydrochloric acid.

14. *In vivo* and *ex vivo* fluorescence imaging.

All the animal experiments were performed in accordance with protocols approved by the Committee for Animal Research of Peking University, China. HT-29 cells were subcutaneously implanted into BALB/c nude female mice on the right flank. CdSe QDs with a fluorescent emission peak at 565 nm were labeled to peptides. After the tumor had developed, QD-labeled STP or TP (200 μL with a concentration of 1 mg/ml) was intravenously injected into an athymic nude mouse bearing a subcutaneous HT-29 cell derived xenograft tumor. The mice treated with only QDs was used as a negative control. Fluorescence Imaging was carried out after 2 h, 4 h, 8 h, 12 h and 24 h using a Maestro in vivo spectrum imaging system. Then animals were sacrificed and tumor, heart, liver, spleen, lung and kidney were excised for fluorescence intensity measurement. To trace the location of the Dye-labeled peptides and evaluation of the vasculature-targeting efficacy, tumors were fixed in 4% paraformaldehyde overnight at 4 °C, then the samples were sent to the company to cut into 6 μm sections for histological analysis. For immunostaining, the sections were dewaxed in dimethylbenzene for 1h at room temperature twice. Then the sections were washed in the 100%, 90%, 80%, 70% gradient alcohol for 5 min, respectively. After drying the sections, they were incubated with the FITC-tagged CD31 antibody overnight in a humidified chamber at 4°C in the dark (1:100 PBS). The sections were washed three times with water for 2 min each time and stained with Cy5-labeled STP or TP at 37°C in the dark for 2h. Finally, the sections were washed three times with water and the nuclei were labeled with Hoechst 33342. Sections were then washed in the 70%, 80%, 90%, 100% gradient alcohol for 5 min, respectively, and last observed using ZEISS LSM710 confocal microscope.

15. The high resolution microscope images of Figure 1 and Figure 2.

Limited by layout of the communication, we show the high resolution cell microscope

images in Figure1 and Figure 2 in order to evaluate the sizes of the cells.

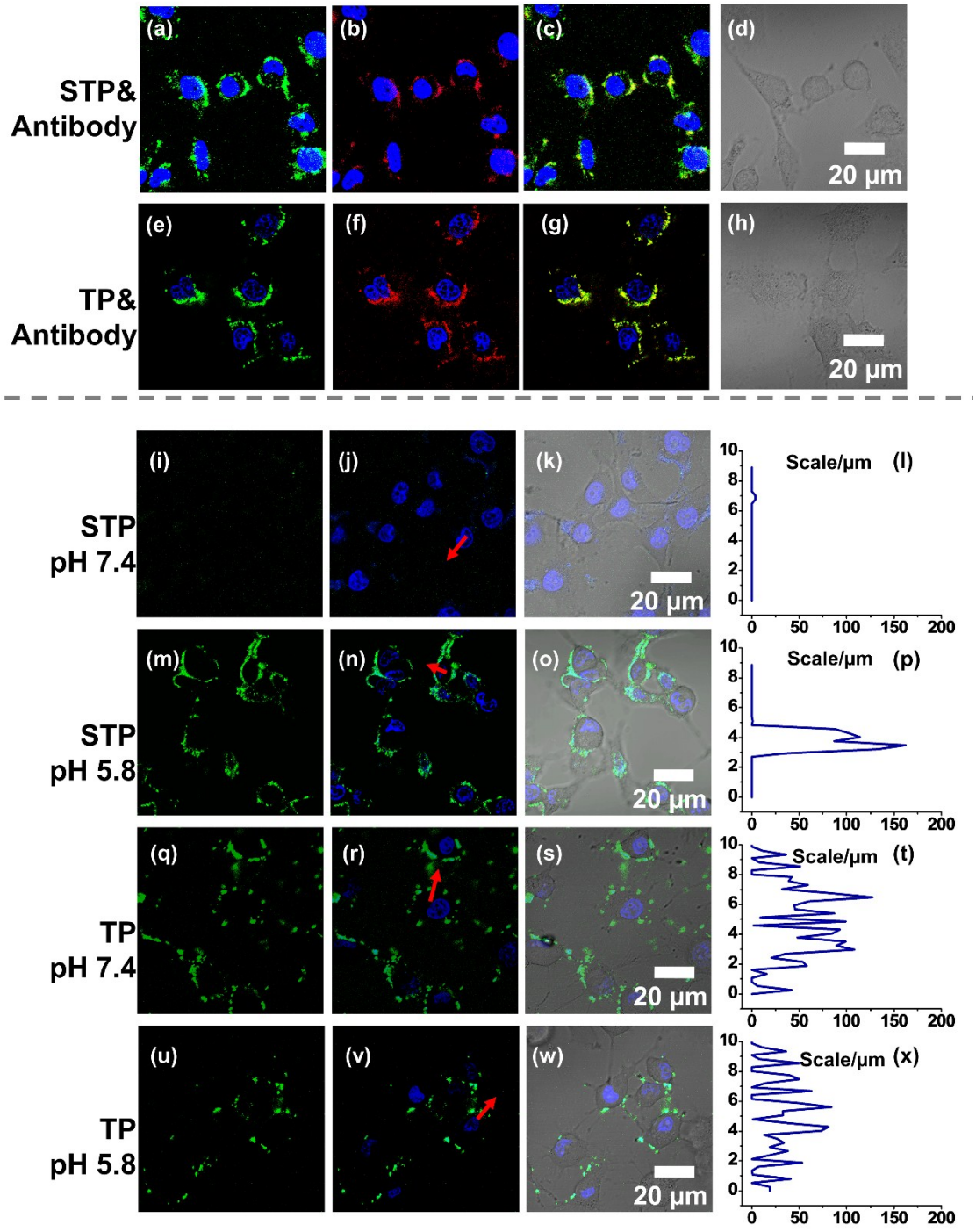

Figure S13. High resolution cell microscope images in Figure1.

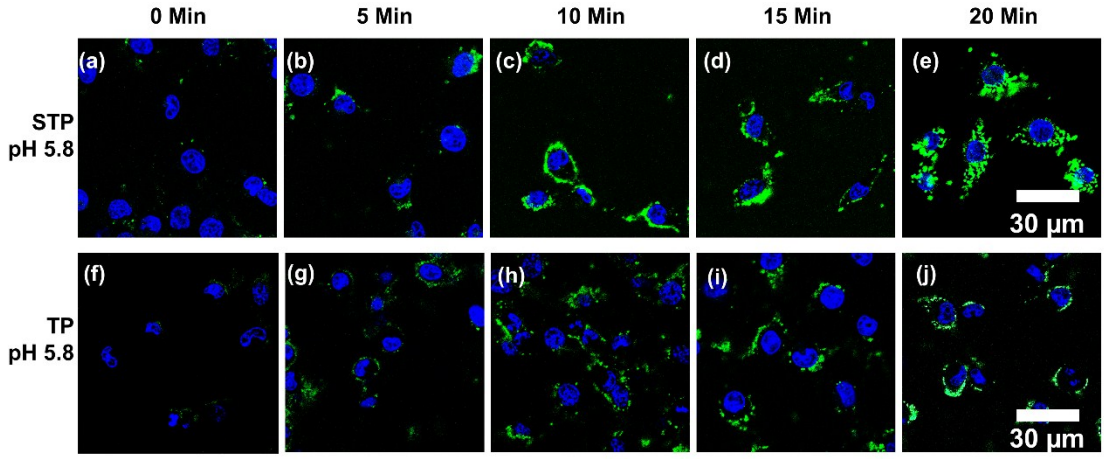

Figure S14. High resolution cell microscope images in Figure2.

References

1. V. M. Leppanen, A. E. Prota, M. Jeltsch, A. Anisimov, N. Kalkkinen, T. Strandin, H. Lankinen, A. Goldman, K. Ballmer-Hofer and K. Alitalo, *Proc. Natl. Acad. Sci. USA*, 2010, **107**, 2425-2430.