Supporting Information for:

Sensitive Detection of Protein Kinase A Activity in Cell Lysates by Peptide Microarray-Based Assay

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Additional Experimental Section

Additional Tables: S1 and S2

Additional Figures: S1-S12

Additional References

Additional Experimental Section

Preparation of AuNP Probes. The citrate stabilized 13 nm AuNPs were synthesized by the traditional Turkevich-Frens method.[S1,S2] Avidin stabilized 13 nm AuNPs (named as AuNP probes) were prepared by stirring an aqueous mixture of succinylated avidin (200 μL, 1 mg/mL in PBS (pH 7.5, 50 mM PB, 0.15 M NaCl)) and citrate stabilized 13 nm AuNPs (2 mL, 3.8 nM) for 30 min at room temperature.[S3] Excess protein was removed by repeated centrifugation at 13000 rpm (16100 g, 3 times) using an Eppendorf centrifuge (Eppendorf, Germany). The as-prepared AuNP probes were redispersed in PBS and stored at 4°C.

Preparation of Cell Lysates. SHG-44 (human glioma cell), HeLa (human cervical cancer cell), MCF-7 (human breast adenocarcinoma cell), SW-620 (human caucasian colon adenocarcinoma cell), and PC-12 (rat adrenal medulla pheochromocytoma cell) cells were grown with fresh medium DMEM supplemented with 10% FBS in a humidified 5% CO_2 incubator for 24 h at 37 °C. The cell lysates were prepared by literature reported method.[S4] Generally, cells (10^6 cells) in 0.3 mL lysis buffer (10 mM potassium phosphate (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT, 1 mM sodium orthovanadate, 80 mM b-glycerophosphate, 3 μg/mL pepstatin A, 5 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) were sonicated for 15 s at 0 °C (3 times) by a JY88-IIN homogenizer (Ningbo Xinyi Ultrasonic Equipment Ltd., China). The homogenates were centrifuged at 13 000 rpm (16100 g) for 50 min at 4 °C using an Eppendorf centrifuge. Then, supernatants were extracted and stored at 0 °C. The pellets were resuspended in 0.2

mL lysis buffer supplemented with 0.1% Triton X-100, and sonicated for 15 s at 0 °C (3 times), centrifuged at 13 000 rpm (16100 g) for 30 min at 4 °C. Subsequently the supernatants were collected and mixed with previously extracts, respectively. Finally, the supernatants (cell lysates) were diluted by desired volumes of PKA assay buffer (50 mM Tris-HCl, 100 mM $MgCl_2$, 0.2 mM ATP, pH 7.5) for using in phosphorylation experiments, respectively. Total proteins of cell lysates were determined using the Bradford reagent. The concentration of cell lysate was defined by the amount of total proteins in the assay solution.

Fabrication of Peptide Microarray. Peptide microarrays were manufactured by the standard procedure using a SmartArrayer 48 system.[S3,S5,S6] Peptides in spotting solution (pH 8.5, 0.3 M PB, 0.2 M NaCl containing 20 μg/mL BSA) with desired concentration were spotted on aldehyde 3-D slides, and incubated under vacuum at 30 °C for 12 h. The slides were washed with 30 mL PB (pH 7.5, 50 mM) containing 1% (w/v) BSA for 5 min (2 times), and then immersed in blocking buffer (pH 7.5, 50 mM PB, 0.15 M NaCl containing 1% w/v BSA and 0.1 M ethanolamine) for 1 h to inactivate any free aldehyde groups on slide surfaces. After incubation with blocking buffer (pH 7.5, 50 mM PB, 0.15 M NaCl containing 1% w/v BSA and 0.1 M ethanolamine) for 1 h, the slides were washed with 30 mL washing buffer (pH 7.5, 20 mM tris, 0.15 M NaCl, 10 mM EDTA, 1 mM EGTA with 0.1% Triton X-100) for 10 min (3 times) and 30 mL kinase buffer (pH 7.5, 50 mM tris, 10 mM $MgCl_2$, 1 mM DTT) for 10 min.

Recognition of Phosphorylation and Attachment of AuNP Probes. After

phosphorylation reaction, the slides were incubated with 200 μL anti-phosphoserine-biotin antibody solution (20 μg/mL in of probe buffer (pH 7.5, 50 mM PB, 0.15 M NaCl, supplemented with 0.1% Tween-20 (v/v) and 1% BSA (w/v)) for 1 h at 37 °C, respectively. Then, a series of washing steps were applied to the slides: (1) 30 mL PBS buffer with 1% Tween-20 for 5 min (3 times), (2) 30 mL PBS buffer for 5 min (3 times), and (3) 30 mL water for 3 min (3 times), respectively. Subsequently, the slides were incubated with 200 μL of AuNP probes (5 nM) in probe buffer for 2 h at 25 °C, respectively. The slides were subjected to a series of washing and drying steps as previously described, respectively.

Silver Enhancement and Detection. 1 mL silver enhancer reagent (1:1 mixed solutions A ($AgNO_3$) and B (hydroquinone)) was applied to each slide for 8 min after being labeled with AuNP probes. Then, slide was washed with 30 mL water (3 times), and dried with centrifugation. After signal amplification by silver deposition, the microarrays were read by an ArrayIt SpotWare Colorimetric Microarray Scanner (TeleChem. International Inc. USA). The background originating from the slide was recorded and subtracted from each image prior to evaluation. The mean values and standard deviations of the signals were determined for the 20 spot replicates per sample from single assay, respectively. The detection limit was determined to be the concentration where signal/standard deviation = 3 was reached. To determine the linear ranges of the curves, the range of concentrations that best fitted the linear equation y= mx + b were specified. The sensitivity is defined by the slope of PKA calibration line of the assay.

Additional Tables: S1 and S2

Table S1. Peptides and their PKA phosphorylation sites and phosphorylation efficiencies

Name	Sequence	conformation	Potential phosphorylation motifs	Phosphorylation efficiency[d]
CP	CGGALRRAGLG	open chain	-	-
SP1	CGGALRRASLG	open chain	1×RRAS[b]	0.2
SP2	CGGALRRASLGPP[a]	circle	1×RRAS[b]	0.26
SP3	CGGALRRASLGAQ	open chain	1×RRAS[b]	0.15
SP4	CGGALRRASLGAQPP[a]	circle	1×RRAS[b]	0.22
SP5	CGGALRRASLGRRAS	open chain	2×RRAS[b]	0.37
SP6	CGGALRRASLGRRASPP[a]	circle	2×RRAS[b]	0.42
SP7	CGGALRRASLGAQLT	open chain	1×RRAS[b] and 1×T[c]	0.24
SP8	CGGALRRASLGAQLTPP[a]	circle	1×RRAS[b] and 1×T[c]	0.28
SPT1	CGGALRRATLG	open chain	1×T[c]	0.06
SPT2	CGGALRRATLGPP[a]	circle	1×T[c]	0.07

[a]The peptides with PP amino acid residues at C-terminals can form circle conformation; the conformations of SP1 and SP6 were shown in Figure S7 as typical examples. [b]Based on the well-known PKA phosphorylation motif RRXS (Here, X=A; see http://www.cbs.dtu.dk/services/NetPhosK). [c]Probable PKA phosphorylated site. [d]The slope of corresponding peptide calibration line; the concentration of ATP in kinase solution is 50 μM and the amount of PKA is 250 U, respectively.

Table S2. XPS measurement of atomic percentage of elements (C, N, P) in phosphorylated SP1 to SP6, SPT1 and CP.

Name	Sequence	C 1s	N 1s	P 2p
CP	CGGALRRAGLG	71.13	28.87	0
SP1	CGGALRRASLG	66.78	28.44	4.78
SP2	CGGALRRASLGPP	67.19	25.91	6.90
SP3	CGGALRRASLGAQ	69.89	28.53	1.58
SP4	CGGALRRASLGAQPP	69.18	25.93	4.89
SP5	CGGALRRASLGRRAS	61.41	27.58	11.01
SP6	CGGALRRASLGRRASPP	62.79	24.60	12.61
SPT1	CGGALRRATLG	70.73	27.96	1.31

After phosphorylation reactions, the slides were studied by X-ray photoelectron spectroscopy (XPS). The XPS experiments were carried out on a Thermo ESCALAB 250 instrument equipped with a monochromatic Al Kα X-ray source (1486.6 eV). Compositional surveys were acquired using a pass energy of 100 eV. High-resolution spectra (N 1s, C 1s, P 2p) were acquired using a pass energy of 20 eV.

The experimentally measured ratios of C to N are consistent with the theoretical values. The P 2p peaks are following the order: SP6>SP5>SP2>SP4>SP1>SP3>SPT1>CP. These results give further evidence on the phosphorylation efficiencies of the peptides.

Additional Figures: S1-S12

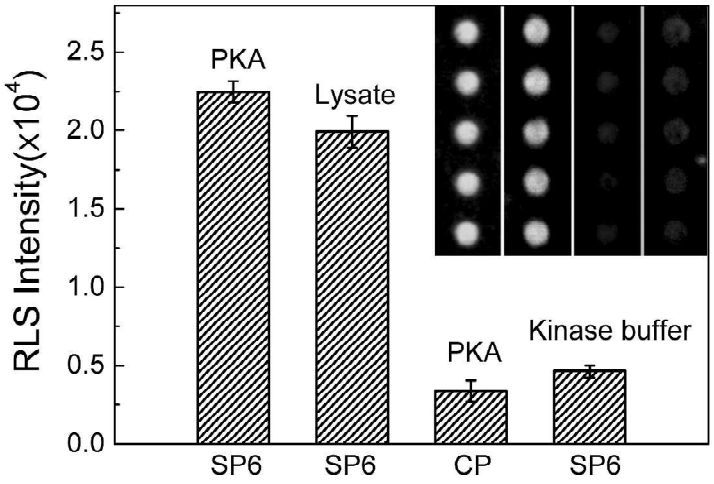

Figure S1. RLS images (inset) and corresponding RLS intensities of SP6 and CP (control peptide) reacted with pure PKA, SP6 reacted with SHG-44 cell lysate and kinase buffer (blank sample), respectively. The concentration of peptides in spotting solution is 10 μM, the concentration of ATP is 50 μM, the amount of PKA is 250 U, and the concentrations of cell lysates are 100 μg/mL, respectively.

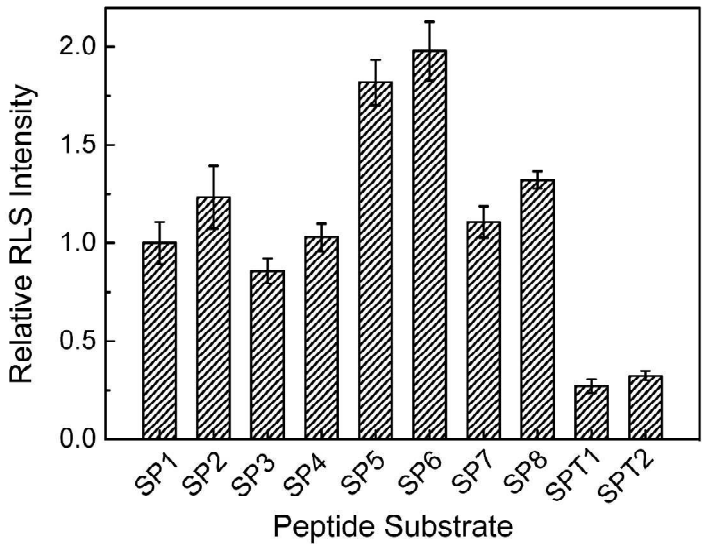

Figure S2. The preliminary quantitative analysis of RLS signals from the spots of 10 peptide substrates. The concentration of peptide in spotting solution is 10 μM, the concentration of ATP is 50 μM, and the amount of PKA is 250 U, respectively. The signals have been subtracted by the average RLS intensity of CP (as shown in the Figure S1).

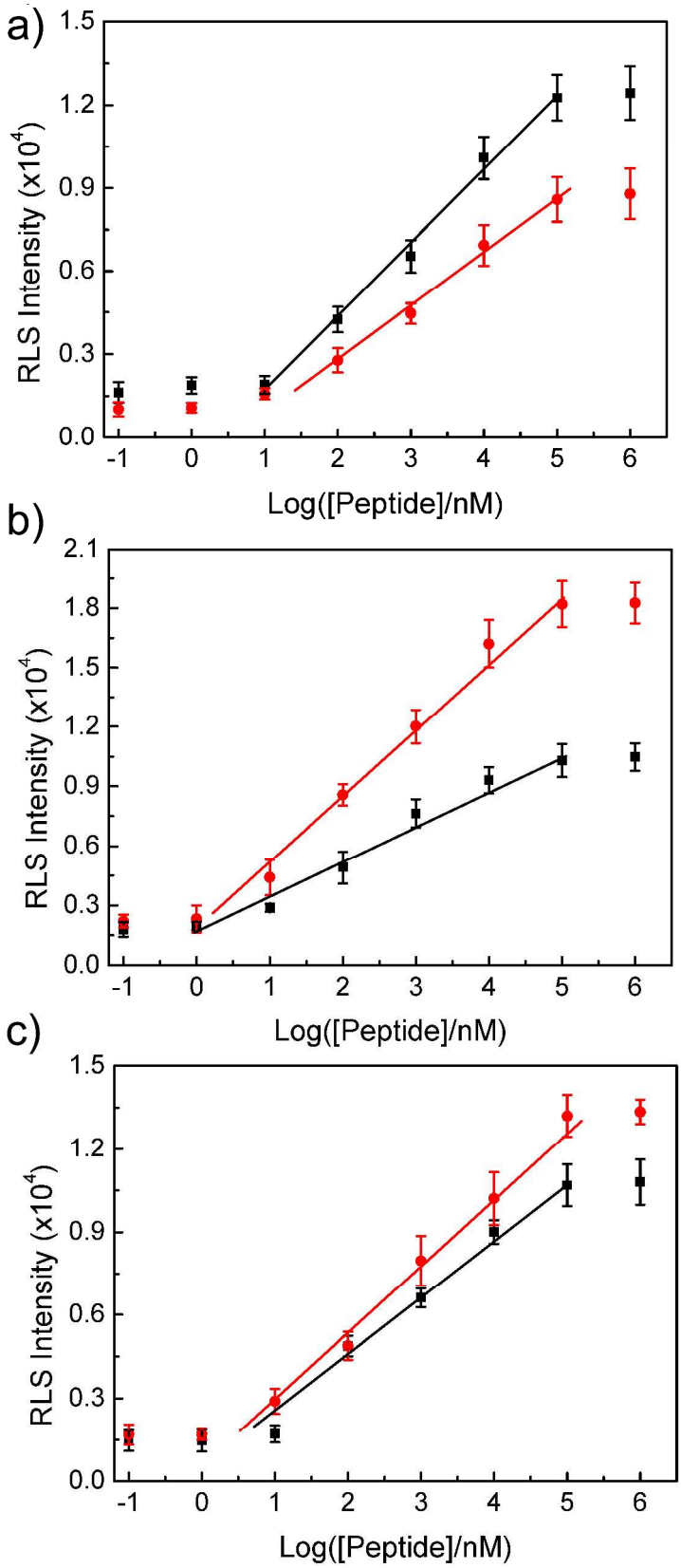

Figure S3. Logarithmic plots of the integrated RLS intensity as a function of the

concentration of SP2 (black square in a), SP3 (red circle in a), SP4 (black square in b), SP5 (red circle in b), SP7 (black square in c) and SP8 (red circle in c), SP9 (black square in d) and SP10 (red circle in d) in the spotting solution. The concentrations of peptides in spotting solution are from 0.1 nM to 1 mM, the concentration of ATP is 50 μM, and the amount of PKA is 250 U, respectively. The signals have been subtracted by the average RLS intensity of CP (as shown in the Figure S1).

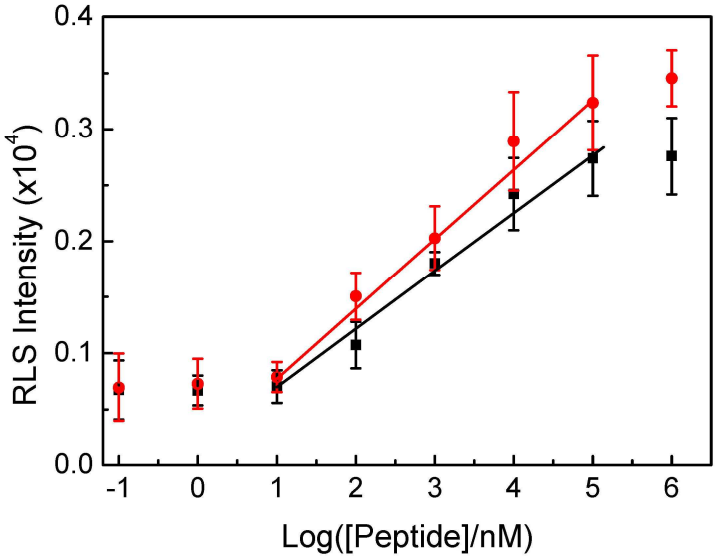

Figure S4. Logarithmic plots of the integrated RLS intensity as a function of the concentration of SPT1 (black square) and SPT2 (red circle) in the spotting solution. The concentrations of peptides in spotting solution are from 0.1 nM to 1 mM, the concentration of ATP is 50 μM, and the amount of PKA is 250 U, respectively. The signals have been subtracted by the average RLS intensity of CP (as shown in the Figure S1).

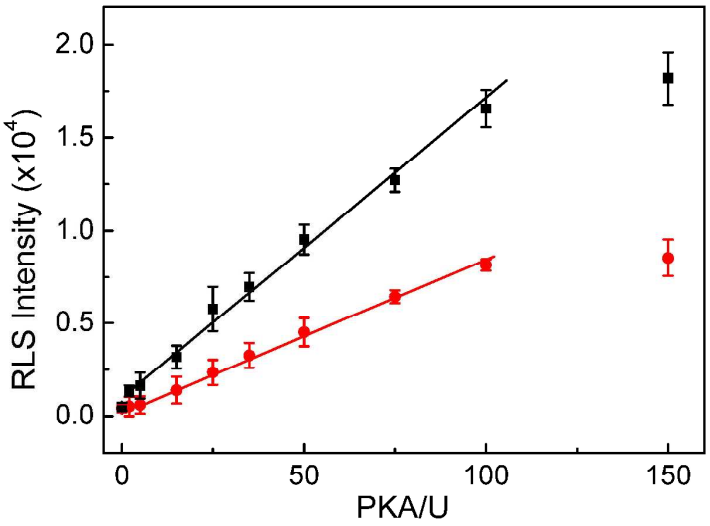

Figure S5. Plots of the integrated RLS intensity as a function of the amount of PKA (0, 2, 5, 15, 25, 35, 50, 75, 100, and 150 U) in the kinase assay buffer. The substrates are SP1 (red circle) and SP6 (black square), respectively. The concentration of SP1 or SP6 in spotting solution is 10 μM, and the concentration of ATP is 50 μM, respectively. The signals have been subtracted by the average RLS intensity of blank sample (as shown in the Figure S1).

The detection limit (0.7 U) of SP6-based assay is less than that of SP1-based assay (5 U), while the sensitivity of SP6-based assay is higher than that of SP1-based assay. The detection limit of SP6-based assay is comparable with the detection limits of the QD-based kinase acitivy assay and peptide microarray-based asaay.[S7,S8] However, the detection limit of SP6-based assay is higher than the detection limits of electrochemical assay S9 and gold nanoparticle-enhanced fluorescence polarization biosensor.[S9,S10]

The PKA assay buffer without pure PKA was employed as blank sample in the phosphorylation reaction process. The detection limit was determined to be the

concentration where signal/standard deviation = 3 was reached. To determine the linear ranges of the curves, the range of concentrations that best fitted the linear equation y= mx + b were specified. The sensitivity is defined by the slope of PKA calibration line of the assay.

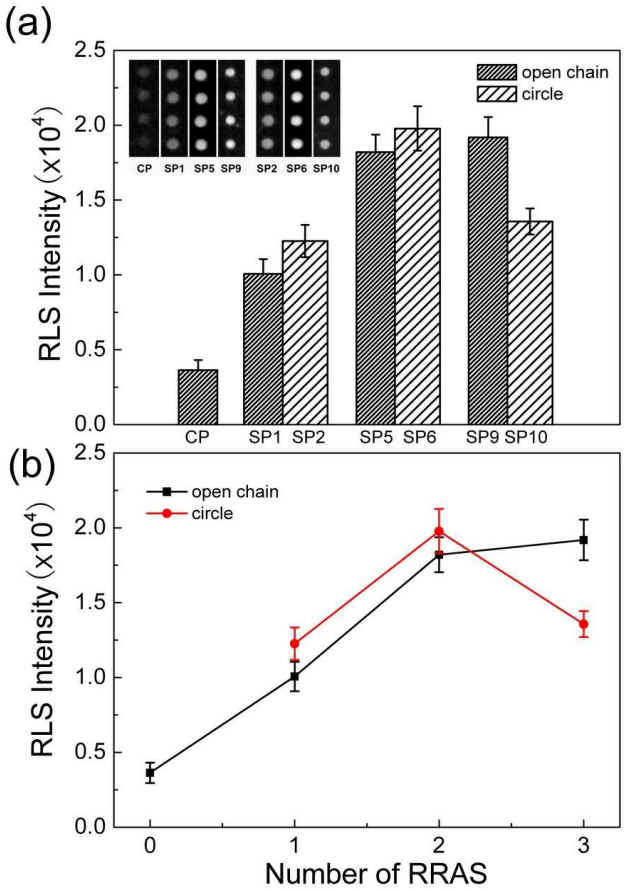

Figure S6. The preliminary quantitative analysis of RLS signals as a function of the numbers of 'RRAS' motifs in peptide substrates. The sequence SP9 (open chain peptide) is CGGALRRASLGRRASLGRRAS and sequence of SP10 (circle chain) is CGGALRRASLGRRASLGRRASPP. The concentrations of peptides in spotting solution are 100 μM, the concentration of ATP is 50 μM, and the amount of PKA is 250 U, respectively.

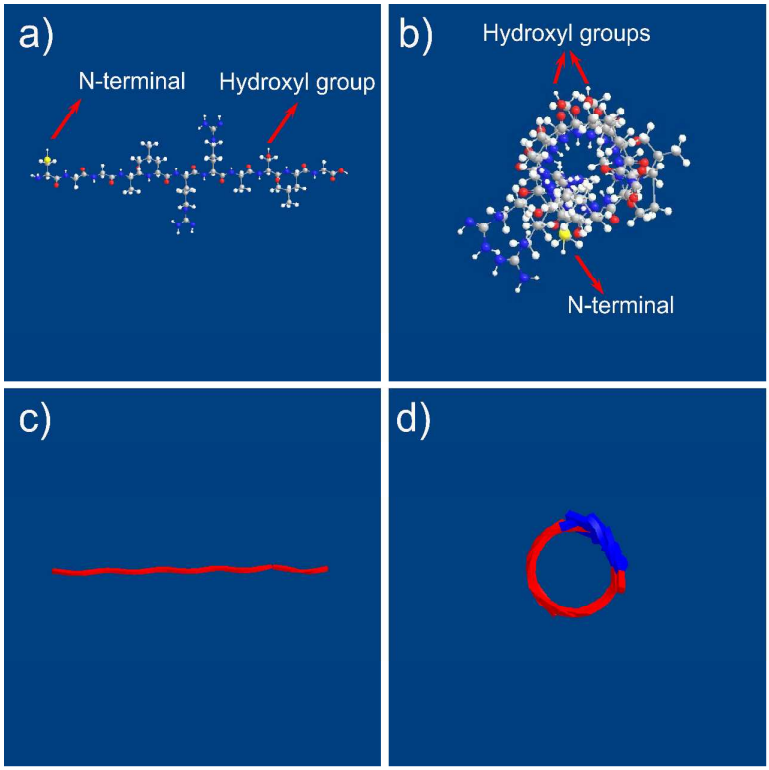

Figure S7. The ball-and-stick models (a and b) and cartoon models (c and d) of the conformations of SP1 (a and c) and SP6 (b and d). SP1 is an open-chain peptide which has one exposed 'RRAS' motif, and SP6 is a cyclic peptide which has two exposed 'RRAS' motifs.

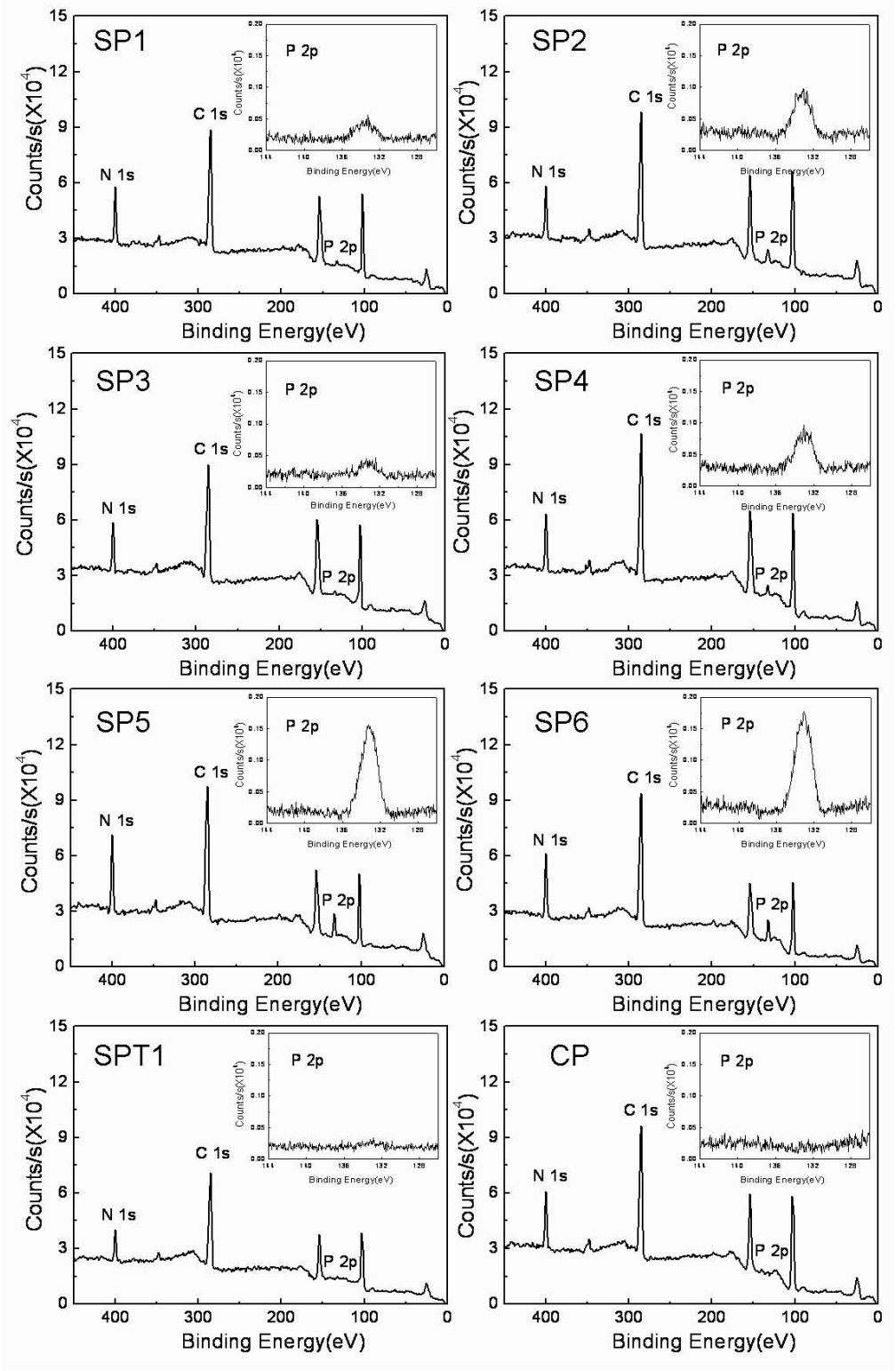

Figure S8. XPS survey spectrums of different substrate peptides after phosphorylation. The insets show the corresponding XPS spectra of P 2p region.

After phosphorylation reactions, the slides were studied by X-ray photoelectron spectroscopy (XPS). The XPS experiments were carried out on a Thermo ESCALAB 250 instrument equipped with a monochromatic Al Kα X-ray source (1486.6 eV). Compositional surveys were acquired using a pass energy of 100 eV. High-resolution spectra (N 1s, C 1s, P 2p) were acquired using a pass energy of 20 eV.

The experimentally measured ratios of C to N are consistent with the theoretical values. The P 2p peaks are following the order: SP6>SP5>SP2>SP4>SP1>SP3>SPT1>CP. These results give further evidence on the phosphorylation efficiencies of the peptides.

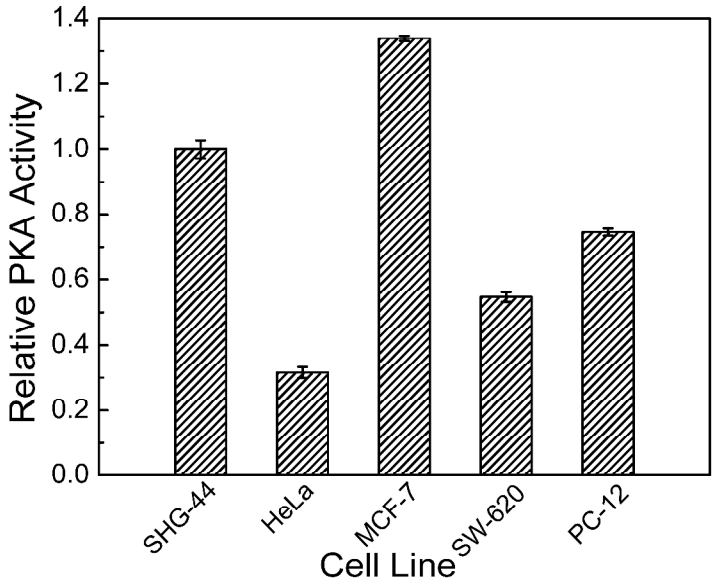

Figure S9. PKA activities of five cell lysates. The concentrations of cell lysates are 100 μg/mL.

PKA activity levels in five cell lysates (SHG-44, HeLa, MCF-7, SW-620 and PC-12,) were detected by commercial PKA ELISA kits (Biofine Ltd. Beijing, China).[S11] Error bars are standard deviations (n=3).

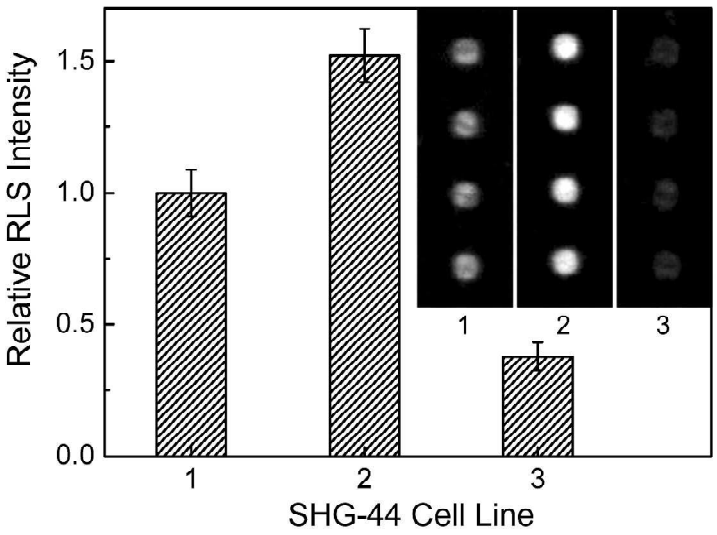

Figure S10. RLS images (inset) and coresponding data analysis on PKA activation/inactivation study. (1) Normal cultured SHG-44 cells, (2) SHG-44 cells co-cultured with 10 μM Fsk, and (3) SHG-44 cells co-cultured with 3 μM H89, respectively. The concentration of SP6 in spotting solution is 10 μM, the concentration of ATP is 50 μM, and the concentrations of SHG-44 cell lysates are 100 μg/mL, respectively. The signals have been normalized to the average RLS intensity of untreated SGH-44 cell lysate.

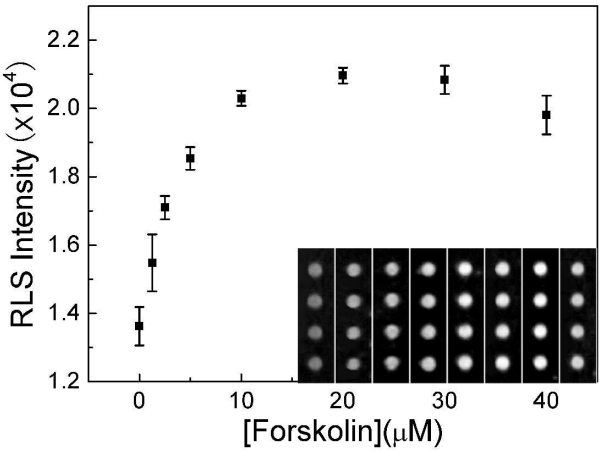

Figure S11. RLS images (inset) and the curve of the integrated RLS intensity as a function of the concentration of Fsk in culture medium. The concentration of SP6 in spotting solution is 10 μM, the concentration of ATP is 50 μM, and the concentrations of SHG-44 cell lysates are 100 μg/mL, respectively. The signals have been subtracted by the average RLS intensity of blank sample (as shown in the Figure S1)

The RLS intensity of Fsk-stimulated SHG-44 cell lysate is increased by increasing the concentration of Fsk from 0 to 20 μM in the culture medium, and then turn to decrease when the concentration of Fsk is over 20 μM.

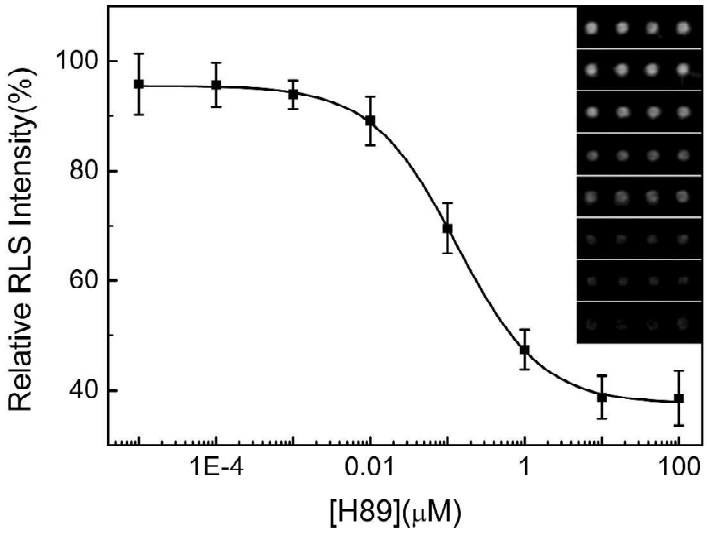

Figure S12. RLS images (inset) and IC_{50} curve of H89. The concentration of SP6 in spotting solution is 10 μM, the concentration of ATP is 50 μM, and the concentrations of SHG-44 cell lysates are 100 μg/mL, respectively. The signals have been normalized to the average RLS intensity obtained in the absence of inhibitor.

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