Supplementary information

Dual fluorescent molecular substrates selectively report the activation, sustainability and reversibility of cellular PKB/Akt activity

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Supplementary Methods

HPLC chromatography. HPLC analysis was performed on a Grace Vydac C-18 column (100 × 4.6 mm) at a flow rate of 1 mL/min. Flow A was 0.1% TFA in water and flow B was 0.1% TFA in acetonitrile. The elution method for analytical HPLC started with 70% A for 3 min, arrived at 30% A in another 18 min, held at 30% A for 3 min, and finally returned to 70% A over one min. The elution profile was monitored by UV absorbance at 254 and 695 nm. Semi-preparative HPLC separation was performed on a Grace Vydac C-18 column (250 × 22 mm) at 23 mL/min.

Synthesis. 3-(3-(1-Carboxy-2-hydroxyethylamino)-3-oxopropyl)-1,1,2-trimethyl-1*H*-benzo[*e*]indolium (**2**): A mixture of compound **1** (362 mg, 1 mmol), **DIC (253** mg, 2 mmol) and HOBT (135 mg, 1 mmol) in DMF (30 mL) was stirred at room temperature for 20 min and then cooled to 0 °C. A solution of *O*-*tert*-butyl-L-serine-*tert*-butyl ester hydrochloride (279 mg, 1.1 mmol) in DMF (10 mL) was added dropwise to the compound **1** mixture. Upon completion of the addition, the resulting reaction mixture was allowed to warm to room temperature and stirred for another 2 h. The reaction solution was concentrated under by vacuum, and the crude product was purified with silica gel column chromatography using a 1:1 hexane:ethyl acetate solution to yield **2c** (526 mg, 94%) as a brown oil. MS $(ESI)^+$ $[M]^+$ calcd 481.3, found 481.2.

3-(3-(1-Carboxy-2-hydroxyethylamino)-3-oxopropyl)-1,1,2-trimethyl-1*H*-benzo[*e*]indolium (**3**): A solution of **2** (56 mg, 0.1 mmol) in 95% TFA (2 mL) was stirred at room temperature for 1 h. The reaction mixture was then concentrated and dried under vacuum. The crude product was collected as dark oil and used in the next step without purification. MS $(ESI)^+$ $[M]^+$ calcd 369.2, found 369.1.

3-(3-(1-Carboxy-2-benzyloxy(hydroxy)phosphoryloxy-ethylamino)-3-oxopropyl)-1,1,2-trimethyl-1*H*-benzo[*e*]indolium (**4**) on solid support: Br-Wang resin (0.3 mmol; 1.4 mmol/g) was pre-swelled in DCM for 40 min, followed by washing with DCM and DMF. A solution of *N*-α-Fmoc-*O*-benzyl-L-phosphoserine (448 mg, 0.9 mmol), DIEA (116mg, 0.9 mmol) and CsI (23.4 mg, 0.09 mmol) in DMF was stirred at room temperature for overnight. The resulting resin was washed with DMF, DCM and hexane to ensure all the reagent, salt and excess amine acids were removed. The resin was dried in vacuum and lyophilized overnight. Fmoc loading of 0.71 mmol/g was determined by absorption spectroscopy.

3-(3-(1-Carboxy-2-(phosphonooxy)ethylamino)-3-oxopropyl)-1,1,2-trimethyl-1*H*-benzo[*e*]indolium (**5**): A solution of **4** (142 mg, 0.1 mmol) in 95% TFA (2 mL) was stirred at room temperature for 1 h. The reaction mixture was then concentrated by vacuum rotary evaporation and dried under vacuum. The crude product was collected as dark oil and used in the next step without further purification. MS $(ESI)^+$ $[M]^+$ calcd 449.1, found 449.1.

Cypate-diserine (**LS456**), cypate-monophospho-diserine (**LS455**) and cypate-diphospho-diserine(**LS631**): A mixture of N-(5-anilino-2,4-pentadienylidene) aniline hydrochloride (1.9 mg, 6.5 μmol) and DIEA (1.7 mg, 13 μmol) in DCM (0.5 mL) was treated with a solution of acetic anhydride (0.8 mg, 7.8 μmol) in DCM (0.5 mL) at 0°C. The resulting clear solution was stirred at room temperature for 1 h. The solvent was removed and replaced with methanol (0.5 mL). The solution was

added to a mixture of **3** (2.4 mg, 6.6 µmol), **5** (2.9 mg, 6.5 µmol), and sodium acetate (2.6 mg, 32 µmol) in methanol (1 mL). The resulting reaction mixture was refluxed overnight, concentrated under vacuum, and purified with preparative HPLC column chromatography using gradient from 70:30 to 30:70 0.1% TFA in acetonitrile: 0.1% TFA in water as eluent. HRMS $(ESI)^+$ $[M]^+$ **LS456**: calcd 799.3707, found 799.3724; **LS455**: calcd 879.3370, found 879.3329. **LS631**: calcd 959.3007, found 959.3212;

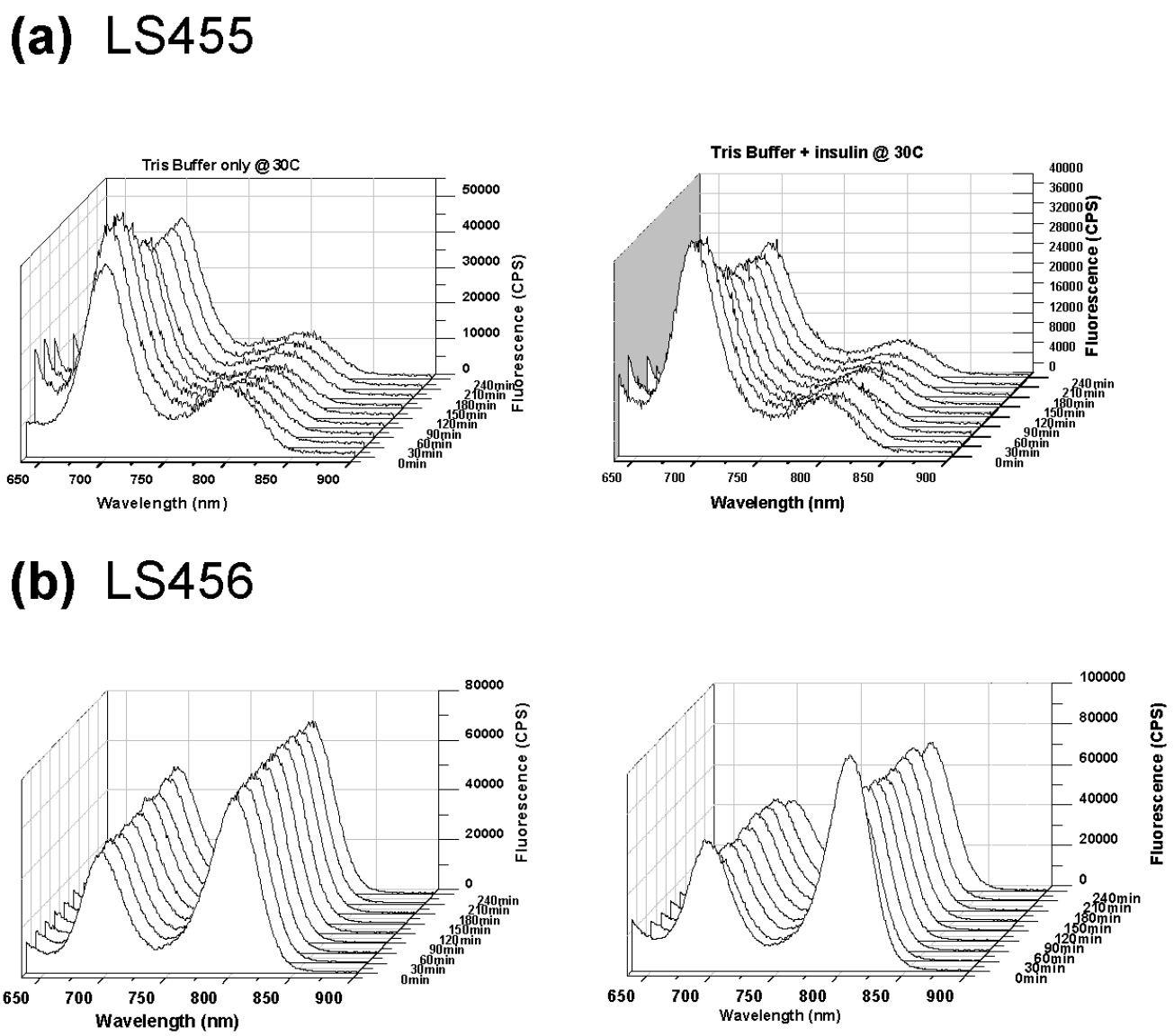
Cypate-tBu-diserine (**LS542**): Cypate was synthesized as previously reported[25]. A mixture of H-Ser(tBu)-OtBu.HCl (0.469 mmol) in DMF (5 mL) was added to a stirring mixture of cypate (0.213 mmol), DIC (0.855 mmol) and HOBT (0.213 mmol) in DMF at 0 °C. The resulting reaction mixture was then warmed to room temperature and stirred for another 4 h. Reaction was monitored by analytical HPLC equipped with VYDAC 218 TP (100 mm x 4.5 mm) C18 column. Flow A was 0.1% TFA in water and Flow B was 0.1% TFA in acetonitrile. The elution method for analytical HPLC started with 40% B over 3 min, attained 100% B in another 17 min, held at 100% B for 3 min and finally returned to 40% B over 1 min. The elution profile was monitored by UV absorption at 254 and 695 nm. DMF was removed, and the crude product was purified by preparative HPLC using a C-18 column (250 × 21.2 mm) at 17 mL/min. The collected solution was concentrated under vacuum, frozen to -78 ºC and dried using a freeze-dry system. LS542 was collected as dark green solid (214 mg, 98%) and its ^{1}H-NMR spectra were measured in $CDCl_3$ at 300 MHz.

Co-immunoprecipitation assay. The compound-transfected MCF-7 cells were treated with 150 nM insulin for 20 or 45 min at 37 ºC and stopped at -80 ˚C for more than 1 h. The cells were collected, homogenized and ultrasonic lysed in cold lysis buffer (2 mM EDTA, 2 mM HEPES) containing complete cocktail protease inhibitors (Roche, Indianapolis, IN). Each homogenized protein was incubated with 50 µg of the monoclonal rabbit anti-phosphorylated Akt (Ser^{473}) (Cell signaling Technology, Inc. Danvers, MA) overnight at 4 °C and pulled down with protein A conjugated magnetic beads (Pierce, Rockford IL) for 2 h. The pellets were rinsed three times with cold PBS (pH 7.4) before separation on 4-12% SDS-PAGE gel (Bio-Rad laboratory, Inc. Hercule, CA). The images were captured with LI-COR Pearl Imager (Lincoln, NE) at Ex/Em of 685/705 nm and 785/810 nm channels. The concentration of protein extracts were determined using spectrometer and separated with 4-12% SDS-PAGE (Bio-Rad). The fluorescence labeled band extracted from polyacrylamid gel for further MS/MS analysis.

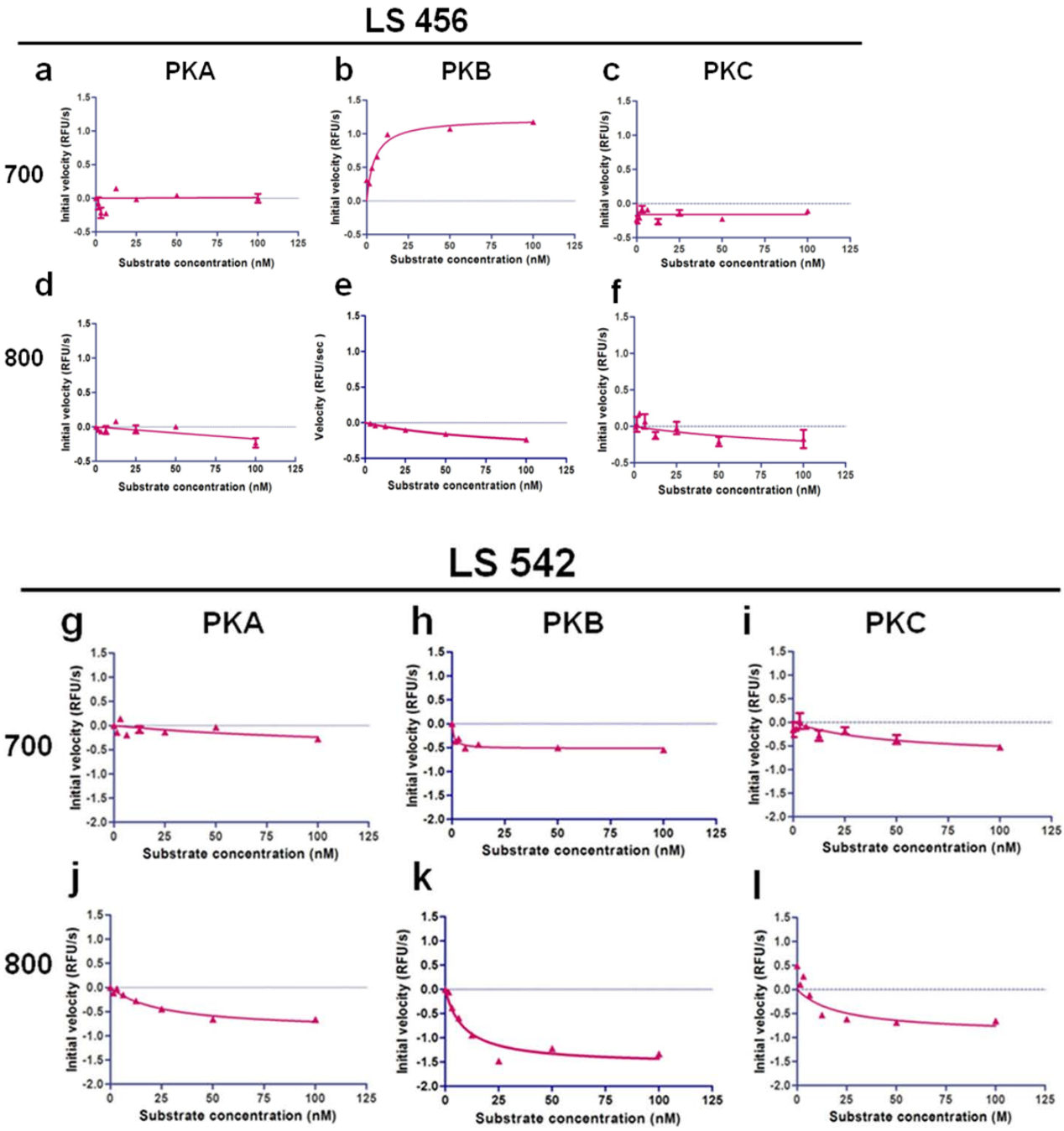
MS/MS mass spectrometry methods. The phosphorylation products were extracted from the gel and analyzed by MS/MS according to the method described by Steinberg et al[1]. Briefly, the bands with the probes were cut from SDS-PAGE gel and washed twice with 50% acetonitrile in deionized water for 20 min and 100% acetonitrile and tried. Peptides and their binding target were subsequently extracted with 50 µL of 10% acetonitrile and 5% TFA for 30 min followed by another 50 µL of 10% acetonitrile and 5% TFA in deionized water for 60 min. All supernatants were dried and dissolved in 15 µL of 10% acetonitrile and 0.1% TFA for MS/MS analysis using TSQ Vantage Mass Spectrometer (Thermo Scientific, San Jose, CA) to identify the phosphorylation products.

Supplementary Figures

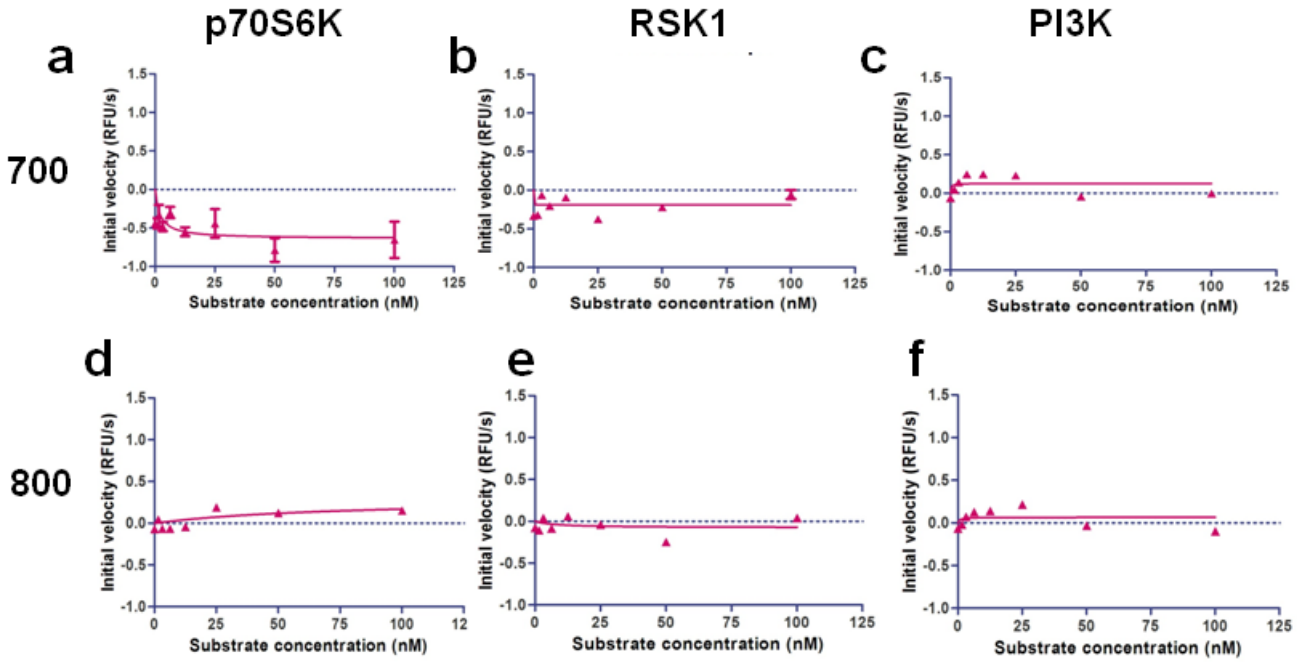
The supplementary figures for the study are shown below.



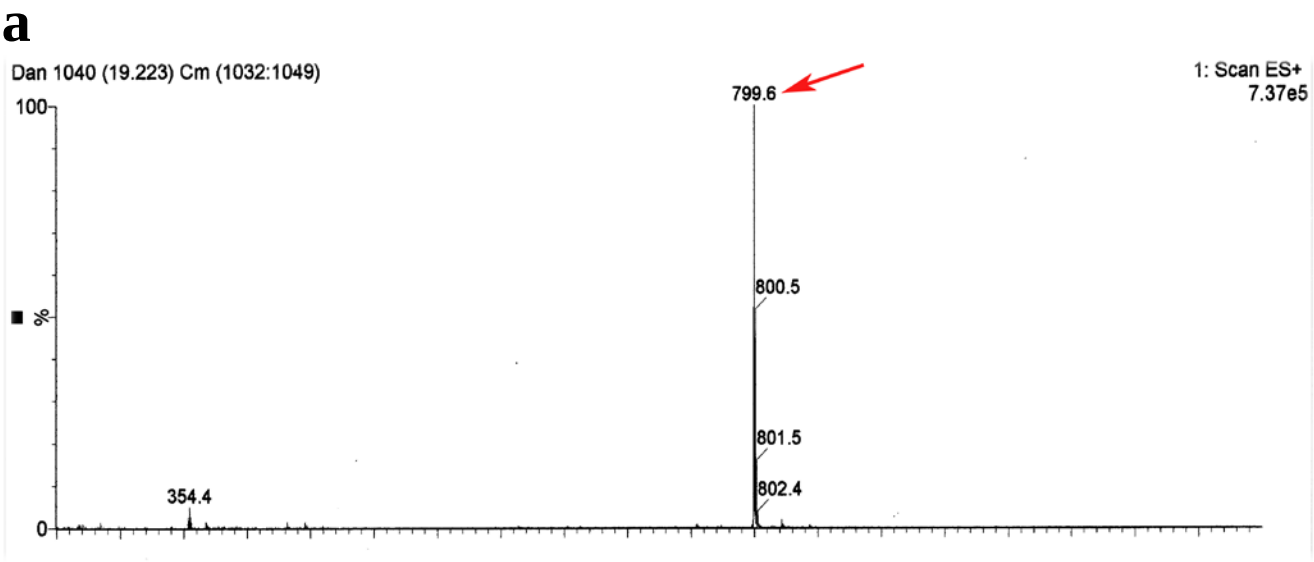
Supplementary Figure 1. Stability testing in vitro. Analysis of the spectral profiles of LS455 (**a**) and LS456 (**b**) in tris saline buffer (TSB; pH 7.4; left panels of **a** and **b**) and insulin dissolved in TSB (pH 7.4; right panels of **a** and **b**) in cell-free system at Ex/Em=620/645-900 nm in 30 ˚C. Although some fluctuations were noted in the spectral profile of LS455 at the early time points, the ratios of the 700/800 nm emissions for LS455 and 456 were stable over time in both TSB and 150 nM insulin in TSB **(**50 mM Tris; 150 mM NaCl; pH=7.4) for 4 h. The minor peak at 650 nm may be caused by the bleed through of the 620 nm excitation light



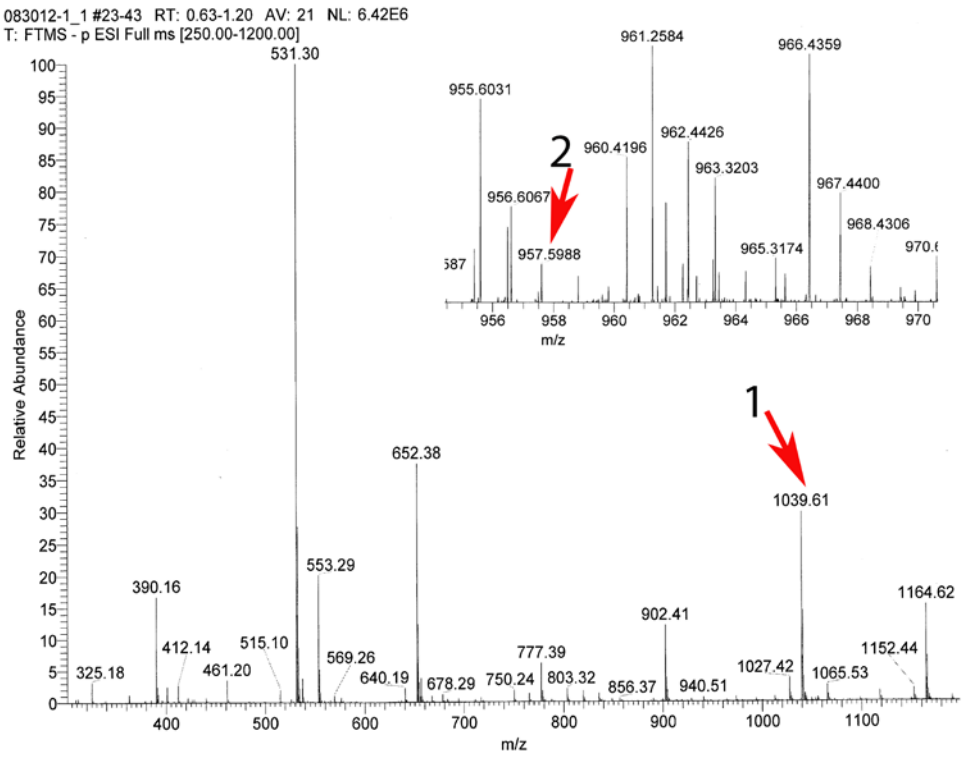
Supplementary Figure 2. Assessment of selective phosphorylation of LS456 (top) and LS542 (bottom) by PKA, PKB, and PKC kinase at 700 nm and 800 nm channels. (**a-f**) Rate of fluorescence change per second vs. substrate concentration at 700 nm (**a-c**) for the incubation of LS456 with PKA (**a**), PKB (**b**), and PKC (**c**) and at 800 nm (**d-f**) with PKA (**d**), PKB (**e**) and PKC (**f**). (**g-i**) Rate of fluorescence change per second vs. substrate concentration at 700 nm (**g-i**) for the incubation of LS542 with PKA (**g**), PKB (**h**), and PKC (**i**) and at 800 nm (**j-l**) with PKA (**j**), and PKB (**k**) and PKC (**i**).



Supplementary Figure 3. Assessment of the phosphorylation of LS456 by activated serine/threonine protein kinases (p70S6K and RSK1) and a tyrosine kinase (PI3K). None of these kinases phosphorylated LS456 under similar conditions as PKB. The consensus substrate motif of p70S6K and RSK1is very similar to that of PKB/Akt, but LS456 was not phosphorylated by these kinases.



b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1039.61
553.29
390.16
1164.62
902.41
412.14
515.10
777.39
1027.42
1152.44
325.18
461.20
569.26
640.19
678.29
750.24
803.32
856.37
940.51
1065.53
961.2584
966.4359
955.6031
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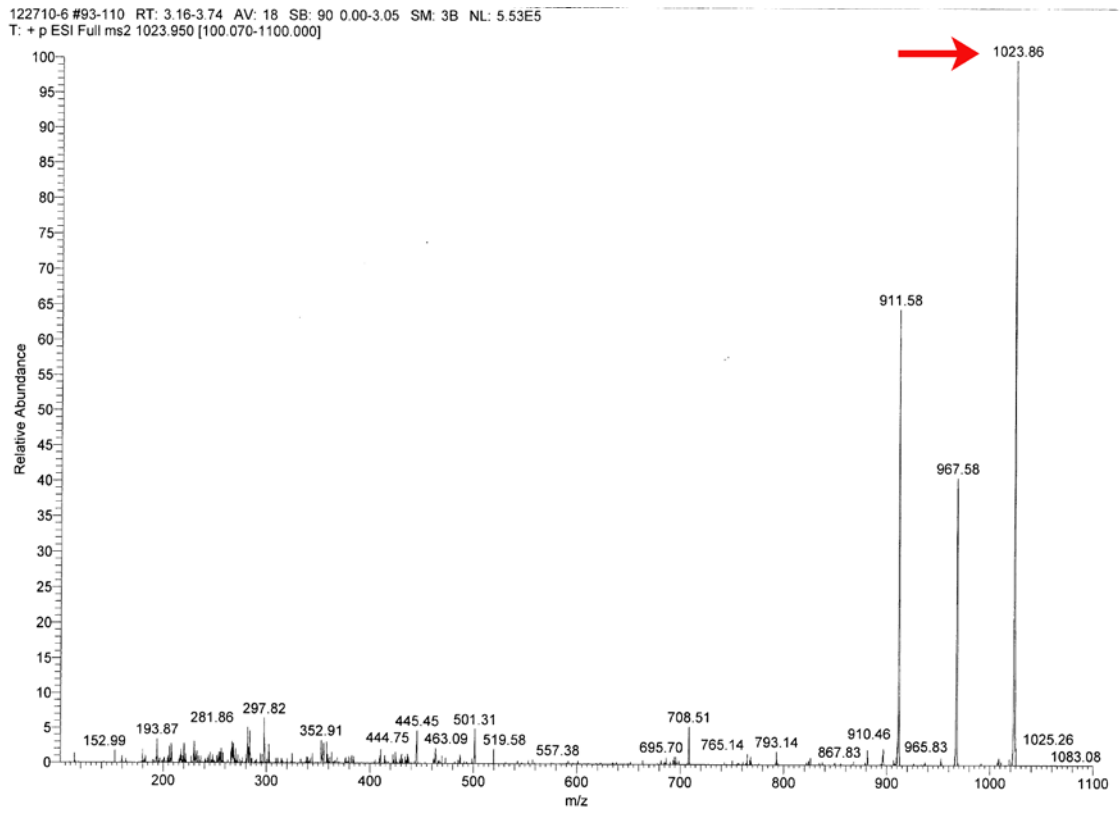
1
2

c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967.58
297.82
281.86
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463.09
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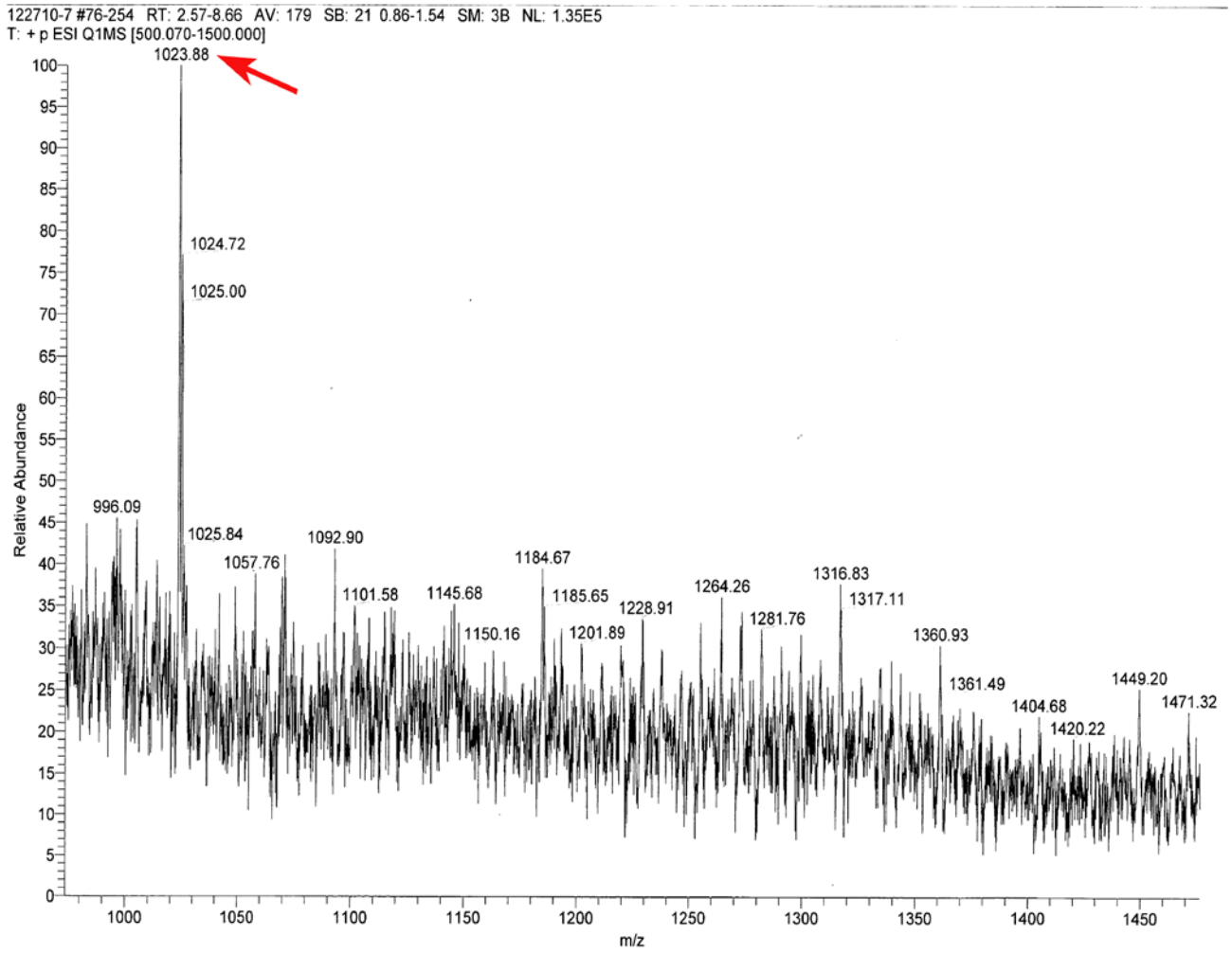
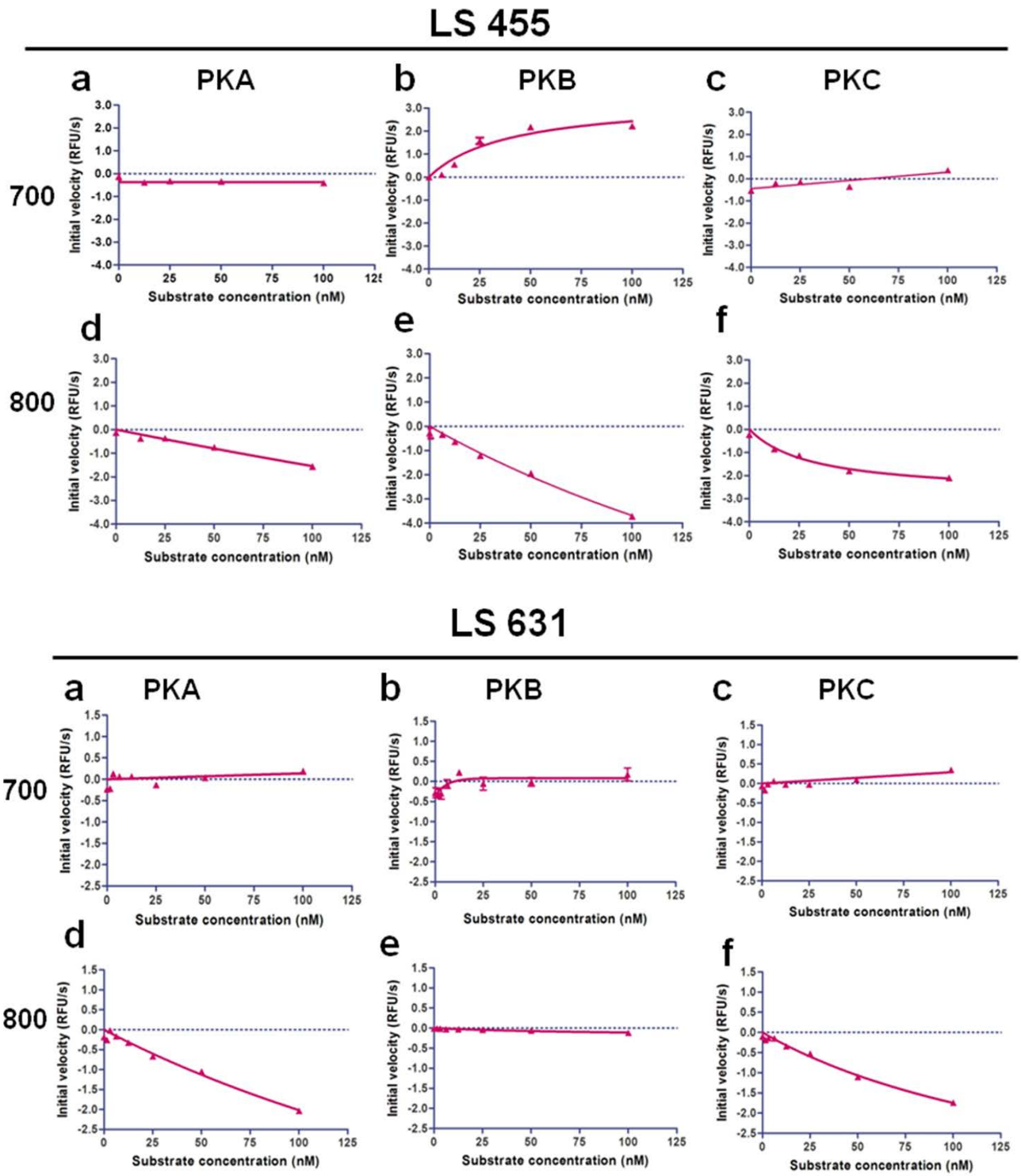
d

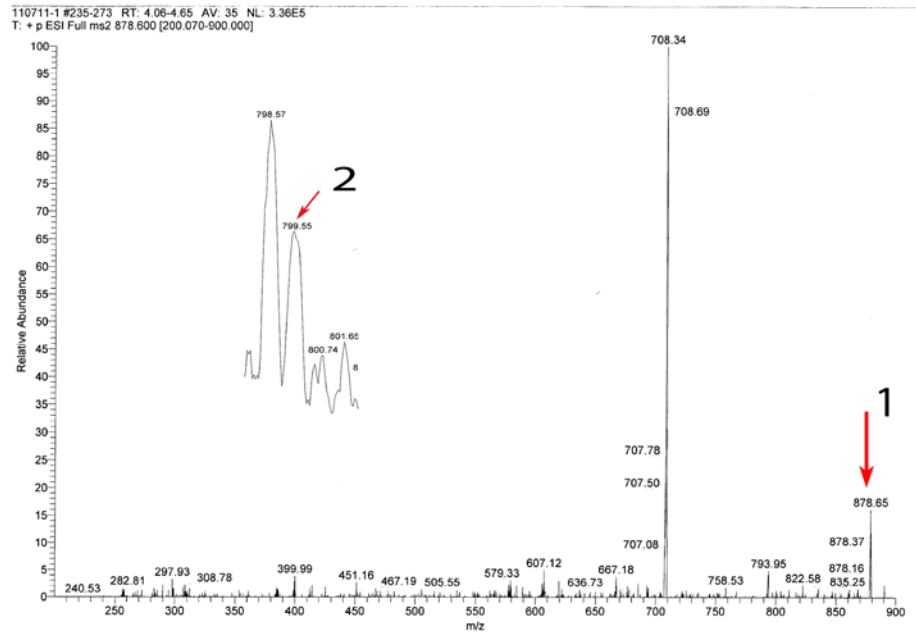


Supplementary Figure 4. **Mass spectral analysis (MS) of LS456 and LS542 phosphorylation in insulin-treated MCF-7 cells. (a)** MS of LS456 in cell-free system. **(b)** MS of LS456 in MCF-7 cells. After 150 nM insulin treatment for 45 min, the MCF-7 cells were homogenized and Co-IP using monoclonal antibody against phosphorylated Akt (Ser^{473} residue). Tri-phosphorylation of LS456 in MCF-7 cells resulted in the expected at 1039.61Da (Arrowhead 1); very small amount of pyro-phosphorylation of LS456 (Arrowhead 2**)** in MCF-7 cells resulted in the expected M/M-2H ions at 959.59/957.6Da. **(c)** MS of the control compound, LS542 (arrow) in cell free system. LS542 was also double treated in MS (MS/MS), which resulted in a sequential loss of two 56 Da corresponding to $C(CH_3)_3$ from *tert*-butyl protecting group. **(d)** MS of LS542 in MCF-7 cells was similar to the cell-free compound LS542 **(c)**.



Supplementary Figure 5. Assessment of LS455 (top) and LS631 (bottom) by PKA, PKB, and PKC at 700 nm and 800 nm channels. The 700 nm emission significantly increased for LS455 with PKB, accompanied by a decrease of the 800 nm fluorescence.

a



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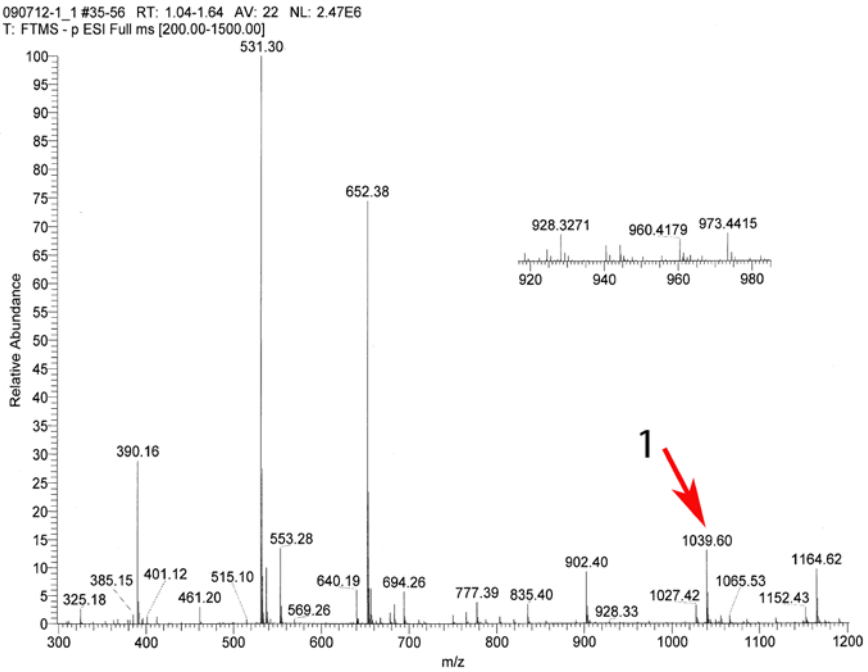
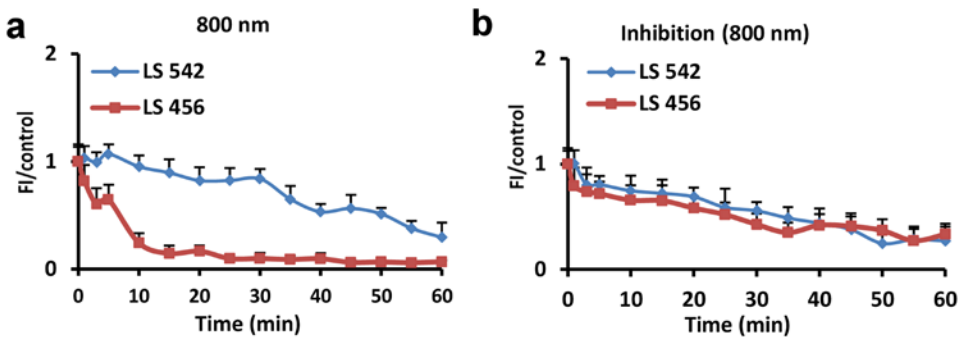
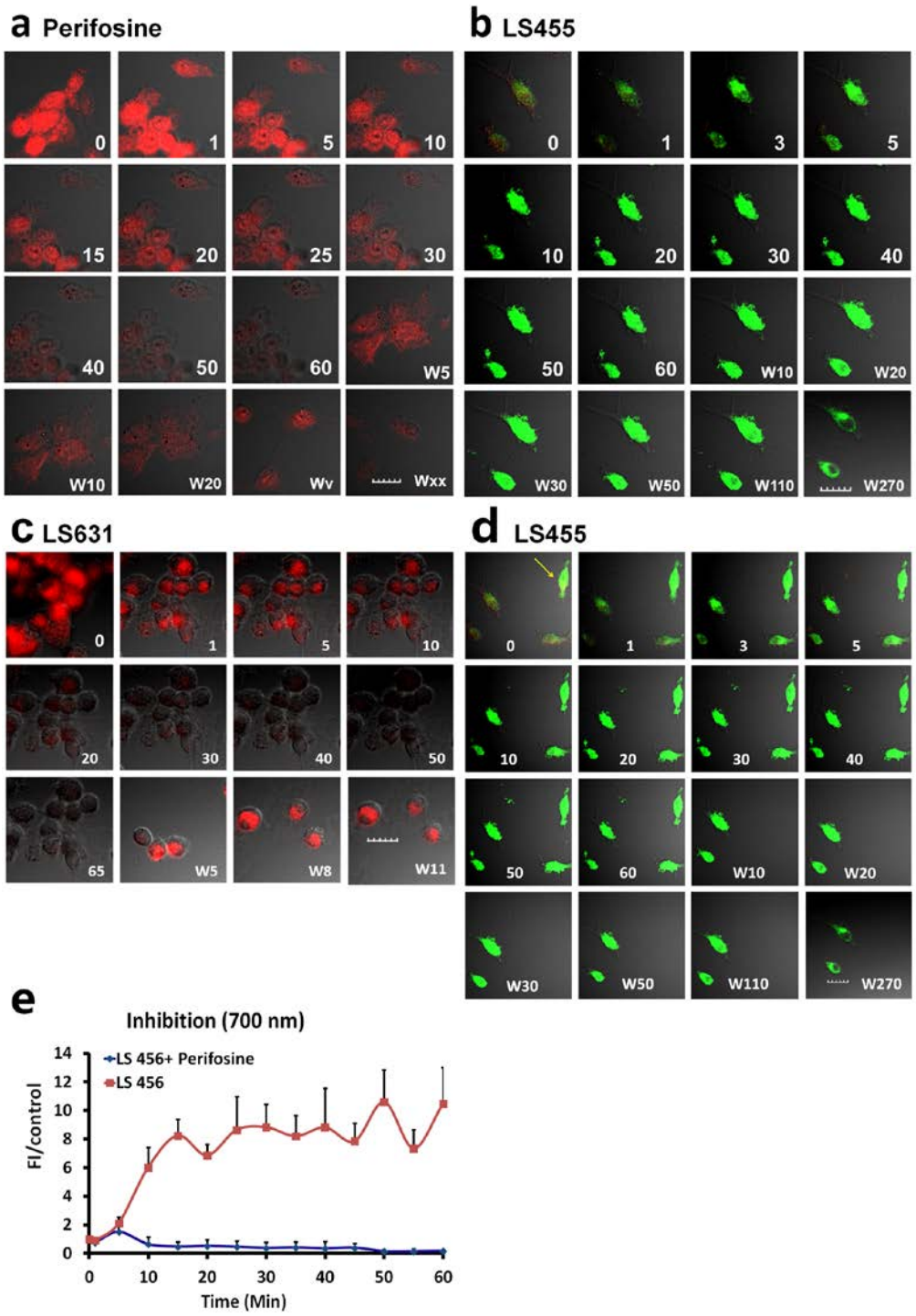


Supplementary Figure 6. Mass spectral analysis of LS455 phosphorylation in insulin-treated MCF-7 cells. (a) MS of LS455 (878.65 Da; arrow 1) in cell-free system. LS455 was double treated in MS, which resulted in a loss of 170 Da, corresponding to $H_2O_3POCH2CHCOOH$ from the monophosphoserine compound**.** There was a small loss of phosphate group in the MS/MS treatment, yielding LS456 with M/M+H ions at 799.55/800.74 (arrow 2). **(b)** MS of LS455 in MCF-7 cells. After 150nM insulin treatment for 45 min, the MCF 7 cells were frozen and homogenized and Co-IP using monoclonal antibody against phosphorylated Akt (Ser^{473} residue). Pyro-phosphorylation of LS455 or tri-phosphorylation of LS456 in MCF-7 cells resulted in the expected M ions at 1039.6Da (Arrowhead 1).



Supplementary Figure 7. Quantitative analysis of dichromic fluorescence change at 800 nm channel in MCF-7 cells. (**a)** The fluorescence of LS456 and LS 542 in the 800 emission channel showing a 10-fold decrease for LS456 within 20 min. The fluorescence change for LS542 is insignificant up to 30 min and decreased gradually, as expected. (**b**) Inhibition of insulin activation of PKB with PI3K inhibitor wortmannin in MCF-7 cells. The rate of fluorescence decrease in the 800 nm emission was about the same for both compounds after inhibition. Error bars are reported as mean ± standard deviation (N=8).



Supplementary Figure 8. Real-time imaging of the effect of insulin and PKB-specific inhibitor (perifosine) on LS455 and LS631. (a) The rapid phosphorylation of LS456 triggered by insulin was inhibited by 50 µM perifosine in the presence of 150 nM insulin after 50 µM perifosine treatment for 2 h. W5: washing with 150 nM insulin in TSB for 5 min. Wv: washing with TSB for 5 min. Wxx: washing with TSB for 20 min. (**b - c**) Imaging of LS455 **(b)** and LS 631 **(c)** with insulin stimulation. **(d)** Zoom out view of panel **(b)**. The arrow points to a cell that cell was washed away at 10 min (W10). All microscopy studies were conducted at 30 ºC. **(e)** Quantitative analysis of panel **(a)** image and comparison with LS456 alone at 700 nm channel. Error bars are reported as mean ± standard deviation (N=8).

Table S1. Spectral properties of LS455, LS456, LS542, and LS631 in PBS. The compounds were dissolved in small amount of DMSO as stock solution. Aliquots were diluted with PBS for the measurements.

Optical Properties	Compounds			
	LS 456	**LS 455**	**LS 542**	**LS631**
Absorption Coefficient at 780 nm ($cm^{-1}M^{-1}$)	137500	135000	142700	138900
Absorption Coefficient at 620 nm ($cm^{-1}M^{-1}$)	7600	8900	8870	7680
Quantum yield 800 nm feature (ex. @620 nm)	2.0%	0.40%	2.1%	2.6%
Quantum yield 700 nm feature (ex. @620 nm)	0.4%	1.2%	0.20%	0.25%

Reference

1. Steinberg, T.H. et al. Global quantitative phosphoprotein analysis using Multiplexed Proteomics technology. *Proteomics* **3**, 1128-44 (2003).