# Supporting Information

# An In-cell Fluorogenic Tag−Probe System for Protein Dynamics Imaging Enabled by Cell-Penetrating Peptides

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**Peptide synthesis**

*General procedure for Fmoc-based solid-phase peptide synthesis*

Peptide synthesis was performed through the Fmoc-based solid-phase peptide synthesis (SPPS). Fmoc-amino acids were coupled by 1,3-diisopropylcarbodiimide (DIPCI, 5.0 eq.) and *N*-hydroxybenzotriazole hydrate (HOBt·$H_2O$, 5.0 eq.). For the coupling of-(nitrobenzo-2-oxa-1,3-diazole) L-α-2,3-diaminopropionic acid, that is, Dap(NBD) to a peptide, 2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU, 2.9 eq.), 1-hydroxy-7-azabenzotriazole (HOAt, 3.0 eq.) and diisopropylethylamine (DIEA, 10 eq.) were employed in place of DIPCI/HOBt. The Fmoc group was deprotected by treatment with 20% (v/v) piperidine/DMF for 20 min. For the fluorescein-labeled peptides, Fmoc-GABA-OH and the subsequent fluorescein were condensed. The resin was extensively washed (DMF, DCM, methanol and diethyl ether) and then dried in vacuo (6 h) before deprotection and cleavage.

*Synthesis of probe **1***

Probe **1** was manually elongated on a Novasyn TGR resin (0.22 mmol/g, 0.1 mmol) by Fmoc-based SPPS. A coupling of Fmoc-amino acids was performed as mentioned above. For the cleavage from the resin and the deprotection, the peptide resin was treated with TFA/*m*-cresol/$H_2O$/triisopropylsilane (TIS) (91.5/2.5/5/1, v/v) for 2 h, followed by filtration. After concentration of the filtrate under reduced pressure, the residue was washed with cold ether and dried under reduced pressure. Purification by reverse phase HPLC gave probe **1** (9.4 mg, 4%). The purified peptide was lyophilized and identified by ESI-TOF-MS recorded on a micrOTOF-2focus (Bruker Daltonics) mass spectrometer; *m*/*z* calcd for $C_{170}H_{314}N_{69}O_{40}$ $[M+H]^+$ 3962.5, found 3962.7.

*Synthesis of probe **2***

Probe **2** was manually elongated on a Novasyn TGR resin (0.22 mmol/g, 0.1 mmol) by Fmoc-based SPPS. A coupling of Fmoc-amino acids was performed as mentioned above. For the cleavage from the resin and the deprotection, the peptide resin was treated with TFA/*m*-cresol/$H_2O$/triisopropylsilane (TIS) (91.5/2.5/5/1, v/v) for 2 h, followed by filtration. After concentration of the filtrate under reduced pressure, the residue was washed with cold ether and dried under reduced pressure. Purification by reverse phase HPLC gave probe **2** (32.5 mg, 11%). The purified peptide was lyophilized and identified by ESI-TOF-MS; *m*/*z* calcd for $C_{164}H_{301}N_{64}O_{40}$ $[M+H]^+$ 3808.5, found : 3806.2.

*Synthesis of probe **3***

Probe **3** was manually elongated on a Novasyn TGR resin (0.22 mmol/g, 0.1 mmol) by

Fmoc-based SPPS. A coupling of Fmoc-amino acids was performed as mentioned above. For the cleavage from the resin and the deprotection, the peptide resin was treated with TFA/*m*-cresol/$H_2O$/triisopropylsilane (TIS) (91.5/2.5/5/1, v/v) for 2 h, followed by filtration. After concentration of the filtrate under reduced pressure, the residue was washed with cold ether and dried under reduced pressure. Purification by reverse phase HPLC gave probe **3** (12.5 mg, 2%). The purified peptide was lyophilized and identified by ESI-TOF-MS; *m*/*z* calcd for $C_{164}H_{302}N_{65}O_{39}$ $[M+H]^+$ 3806.4, found 3806.4.

*Synthesis of fluorescein-conjugated probe **1** (probe **4**)*

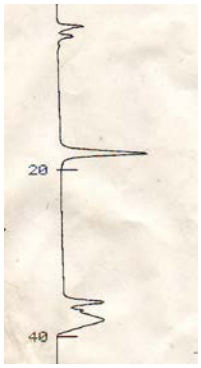
Probe **4** was manually elongated on a Novasyn TGR resin (0.22 mmol/g, 0.05 mmol) by Fmoc-based SPPS. A coupling of Fmoc-amino acids, including Fmoc-GABA and fluorescein, was performed as mentioned above. For the cleavage from the resin and the deprotection, the peptide resin was treated with TFA/*m*-cresol/$H_2O$/triisopropylsilane (TIS) (91.5/2.5/5/1, v/v) for 2 h, followed by filtration. After concentration of the filtrate under reduced pressure, the residue was washed with cold ether and dried under reduced pressure. Purification by reverse phase HPLC gave probe **4** (5.6 mg, 2%). The purified peptide was lyophilized and identified by ESI-TOF-MS; *m*/*z* calcd for $C_{192}H_{329}N_{70}O_{44}$ $[M+H]^+$ 4319.6, found 4319.6.

*Synthesis of fluorescein-conjugated probe **3** (probe **5**)*

Probe **5** was manually elongated on a Novasyn TGR resin (0.22 mmol/g, 0.05 mmol) by Fmoc-based SPPS. A coupling of Fmoc-amino acids, including Fmoc-GABA and fluorescein, was performed as mentioned above. For the cleavage from the resin and the deprotection, the peptide resin was treated with TFA/*m*-cresol/$H_2O$/triisopropylsilane (TIS) (91.5/2.5/5/1, v/v) for 2 h, followed by filtration. After concentration of the filtrate under reduced pressure, the residue was washed with cold ether and dried under reduced pressure. Purification by reverse phase HPLC gave fluorescein-conjugated probe **5** (9.8mg, 3%). The purified peptide was lyophilized and identified by ESI-TOF-MS; *m*/*z* calcd for $C_{186}H_{315}N_{65}O_{44}$ $[M+H]^+$ 4163.5, found 4165.5.

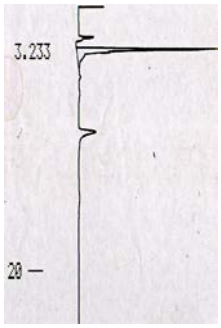
**HPLC charts of probe peptides**

Probe **1**



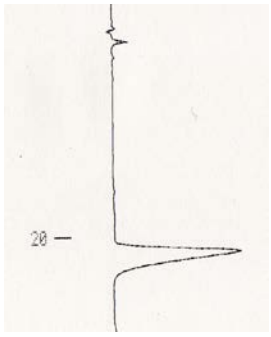

Retention time: 19 min with MeCN (25-35%)

Probe **2**



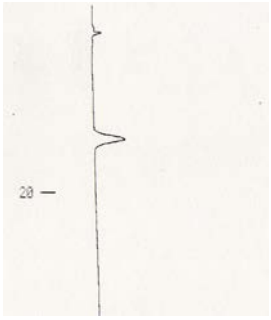

Retention time: 8 min with MeCN (27%, isocratic)

Probe **3**



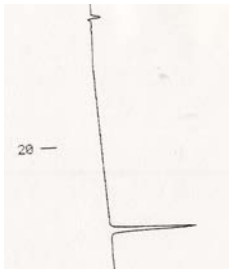

Retention time: 21 min with MeCN (25-28%)

Probe **4**




Retention time: 14 min with MeCN (30-35%)

Probe **5**




Retention time: 29 min with MeCN (25-35%)

**Construction of plasmids for fusion proteins.**

*Construction of mKO-tag gene*

The gene was amplified by PCR utilizing pCIER-tag-flag-mKO as a template.

The PCR primers are as followings:

NheI-tag-Fw

5'-AGA AGA AAG CTA GCA TGG CTC TGA AGA AAG AGC TG-3'

mKO-stop-XbaI-Rv

5'-TCT TCT TTT CTA GAT CAG GAA TGA GCT ACT GCA TCT T-3'

The amplified DNA fragment was cloned into pCI plasmid vector (Promega) as NheI/XbaI fragment resulting pCI-tag-FLAG-mKO.

The amino acid sequence of expressed mKO-tag fusion protein is as followings:

ALKKELEAAKKELEALKKELAGGCGGALEKELEALEKEAEALEKELAGSGSGSGTDMDYKDDDDKEFMVSVIKPEMKM
RYYMDGSVNGHEFTIEGEGTGRPYEGHQEMTLRVTMAKGGPMPFAFDLVSHVFCYGHRPFTKYPEEIPDYFKQAFPEG
LSWERSLEFEDGGSASVSAHISLRGNTFYHKSKFTGVNFPADGPIMQNQSVDWEPSTEKITASDGVLKGDVTMYLKLE
GGGNHKCQFKTTYKAAKKILKMPGSHYISHRLVRKTEGNITELVEDAVAHS

The highlights indicate the sections of sequences; light blue, tag; green, FLAG; red, mKO.

*Construction of ER-mKO-tag gene*

Signal sequence for ER localization was as following.

MLLPVPLLLGLLGAAAD for N-terminal and KDEL for C-terminal of target proteins.

The coding sequences for these signal peptides were amplified by PCR as fused to N- and C-terminal of tag-flag sequence. The amplified DNA fragment was inserted to pCI plasmid vector (Promega) as NheI/XbaI fragment resulting pCIER-tag-flag.

The gene for mKO was amplified from CoralHue® monomeric Kusabira-Orange (pmKO1; MBL). The amplified fragment was inserted to pCIER-tag-flag as EcoRI ftragment resulting pCIER-tag-flag-mKO.

The primers used in this sub-cloning are as followings;

ER-tag Fw-1

5'-TGCTGCTGGGCCTGCTGGGCGCCGCCGCGGATGGAGGTTCTGCTCTGAAGAAAGAGC T-3'

ER-tag Fw-2

5'-GAGGAGGCTAGCATGCTGCTGCCCGTCCCCCTGCTGCTGGGCCTGCTGG-3'

ER-tag Rv+STOP

5'- CTCCTCTCTAGATCACAGCTCGTCCTTGAATTCCTTGTCATCGTCGTCCTTGTA -3'

EcoRI mKO ER Fw

5’-GAGGAGGAATTCATGGTGAGTGTGATTAAACCAGAG-3’

EcoRI mKO ER Rv

5'-CTCCTCGAATTCGGAATGAGCTACTGCATCTTC-3'

The amino acid sequence of expressed ER-mKO-tag is as followings:

MLLPVPLLLGLLGAAADGGSALKKELEAAKKELEALKKELAGGCGGALEKELEALEKEAEALEKELAGSGSGSGTDMD
YKDDDDKEFMVSVIKPEMKMRYYMDGSVNGHEFTIEGEGTGRPYEGHQEMTLRVTMAKGGPMPFAFDLVSHVFCYGHR
PFTKYPEEIPDYFKQAFPEGLSWERSLEFEDGGSASVSAHISLRGNTFYHKSKFTGVNFPADGPIMQNQSVDWEPSTE
KITASDGVLKGDVTMYLKLEGGGNHKCQFKTTYKAAKKILKMPGSHYISHRLVRKTEGNITELVEDAVAHSEFKDEL

The highlights indicate the sections of sequnces; grey, ER-localization signal; light blue, tag; green, FLAG; red, mKO.

*Construction of H2B-mKO-tag gene*

H2B gene was amplified by PCR utilizing pSNAPf-H2B (New England Biolab) as a template.

The PCR primers are as followings:

NheI_Kozak_H2B_Fw

5’-GGGAGGAGGCTAGCGCCACCATGCCAGAGCCAGCGAAG-3’

H2B_HindIII_Rv

5’-CCCTCCTCAAGCTTCTTAGCGCTGGTGTACTTGGT-3’

The amplified DNA fragment was cloned into pcDNA3.1(+) resulting pcDNAH2B.

The gene for tag-FLAG-mKO was amplified from pCIER-tag-flag-mKO by primers as follows:

HindIII_tagFLAGmKO_Fw

5’-GGGAGGAGAAGCTTGCTCTGAAGAAAGAGCTGGAA-3’

tagFLAGmKO_XbaI_Rv

5’-CCCTCCTCTCTAGAGGAATGAGCTACTGCATCTTC-3’

The amplified DNA fragment was cloned into pcDNAH2B as HindIII/XbaI fragment resulting pcDNAH2B tag-FLAG-mKO.

The amino acid sequence of expressed H2B-mKO-tag is as followings:

MPEPAKSAPAPKKGSKKAVTKAQKKGGKKRKRSRKESYSIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFERIAGEA
SRLAHYNKRSTITSREIQTAVRLLLPGELAKHAVSEGTKAITKYTSAKKLALKKELEAAKKELEALKKELAGGCGGAL
EKELEALEKEAEALEKELAGSGSGSGTDMDYKDDDDKEFMVSVIKPEMKMRYYMDGSVNGHEFTIEGEGTGRPYEGHQ
EMTLRVTMAKGGPMPFAFDLVSHVFCYGHRPFTKYPEEIPDYFKQAFPEGLSWERSLEFEDGGSASVSAHISLRGNTF
YHKSKFTGVNFPADGPIMQNQSVDWEPSTEKITASDGVLKGDVTMYLKLEGGGNHKCQFKTTYKAAKKILKMPGSHYI
SHRLVRKTEGNITELVEDAVAHSSRGPV

The highlights indicate the sections of sequnces; Yellow, H2B; light blue, tag; green, FLAG; red,

mKO.

*Construction of PKCδ-mKO-tag gene*

Tag-flag-PKCδ gene was synthesized by custom DNA synthesis by Takara Bio.

The amino acid sequence of the tag-FLAG-PKCδ is as following:

MALKKELEAAKKELEALKKELAGGCGGALEKELEALEKEAEALEKELAGSGSGSGTDMDYKDDDDKGSMAPFLRIAFN
SYELGSLQAEDEANQPFCAVKMKEALSTERGKTLVQKKPTMYPEWKSTFDAHIYEGRVIQIVLMRAAEEPVSEVTVGV
SVLAERCKKNNGKAEFWLDLQPQAKVLMSVQYFLEDVDCKQSMRSEDEAKFPTMNRRGAIKQAKIHYIKNHEFIATFF
GQPTFCSVCKDFVWGLNKQGYKCRQCNAAIHKKCIDKIIGRCTGTAANSRDTIFQKERFNIDMPHRFKVHNYMSPTFC
DHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLCGINQKLLAEALNQVTQRASRRSDSASSEPVGIYQGFEKKTGV
AGEDMQDNSGTYGKIWEGSSKCNINNFIFHKVLGKGSFGKVLLGELKGRGEYFAIKALKKDVVLIDDDVECTMVEKRV
LTLAAENPFLTHLICTFQTKDHLFFVMEFLNGGDLMYHIQDKGRFELYRATFYAAEIMCGLQFLHSKGIIYRDLKLDN
VLLDRDGHIKIADFGMCKENIFGESRASTFCGTPDYIAPEILQGLKYTFSVDWWSFGVLLYEMLIGQSPFHGDDEDEL
FESIRVDTPHYPRWITKESKDILEKLFEREPTKRLGVTGNIKIHPFFKTINWTLLEKRRLEPPFRPKVKSPRDYSNFD
QEFLNEKARLSYSDKNLIDSMDQSAFAGFSFVNPKFEHLLEDEFGGG

The highlights indicate the sections of sequnces; light blue, tag; green, FLAG; pink, PKC.

The gene was amplified by PCR with the primers.

NheItagFLAGPKCF

5'-GGAGAAGAGCTAGCGCCACCATGGCTCTGAAGAAAGAGCTG-3'

tagFLAGPKCEcoRIR

5'-CCTCTTCTGAATTCATCTTCCAGGAGGTGCTCGAA-3'

The PCR fragment was gel purified and inserted into pcDNA 3.1 (+) as NheI/EcoRI fragment resulting pcDNAtagFLAGPKC.

The mKO gene was amplified by PCR with primers.

EcoRIGlymKOF

5'-GGAGAAGAGAATTCGGAGGGGGTATGGTGAGTGTGATTAAACCAG-3'

mKOXbaISTOPRv

5'-CCTCTTCTTCTAGACTAATGAGCTACTGCATCTTCTAC-3'

The PCR fragment was gel purified and inserted to pcDNAtagFLAGPKC as a EcoRI/XbaI fragment resulting pcDNAtagFLAGPKC-mKO.

The amino acid sequence of the PKCδ-tag-mKO is as following:

MALKKELEAAKKELEALKKELAGGCGGALEKELEALEKEAEALEKELAGSGSGSGTDMDYKDDDDKGSMAPFLRIAFN
SYELGSLQAEDEANQPFCAVKMKEALSTERGKTLVQKKPTMYPEWKSTFDAHIYEGRVIQIVLMRAAEEPVSEVTVGV
SVLAERCKKNNGKAEFWLDLQPQAKVLMSVQYFLEDVDCKQSMRSEDEAKFPTMNRRGAIKQAKIHYIKNHEFIATFF
GQPTFCSVCKDFVWGLNKQGYKCRQCNAAIHKKCIDKIIGRCTGTAANSRDTIFQKERFNIDMPHRFKVHNYMSPTFC

DHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLCGINQKLLAEALNQVTQRASRRSDSASSEPVGIYQGFEKKTGV
AGEDMQDNSGTYGKIWEGSSKCNINNFIFHKVLGKGSFGKVLLGELKGRGEYFAIKALKKDVVLIDDDVECTMVEKRV
LTLAAENPFLTHLICTFQTKDHLFFVMEFLNGGDLMYHIQDKGRFELYRATFYAAEIMCGLQFLHSKGIIYRDLKLDN
VLLDRDGHIKIADFGMCKENIFGESRASTFCGTPDYIAPEILQGLKYTFSVDWWSFGVLLYEMLIGQSPFHGDDEDEL
FESIRVDTPHYPRWITKESKDILEKLFEREPTKRLGVTGNIKIHPFFKTINWTLLEKRRLEPPFRPKVKSPRDYSNFD
QEFLNEKARLSYSDKNLIDSMDQSAFAGFSFVNPKFEHLLEDEFGGGMVSVIKPEMKMRYYMDGSVNGHEFTIEGEGT
GRPYEGHQEMTLRVTMAKGGPMPFAFDLVSHVFCYGHRPFTKYPEEIPDYFKQAFPEGLSWERSLEFEDGGSASVSAH
ISLRGNTFYHKSKFTGVNFPADGPIMQNQSVDWEPSTEKITASDGVLKGDVTMYLKLEGGGNHKCQFKTTYKAAKKIL
KMPGSHYISHRLVRKTEGNITELVEDAVAH

The highlights indicate the sections of sequnces; light blue, tag; green, FLAG; pink, PKCδ; red, mKO.

**Supporting Information Figures**

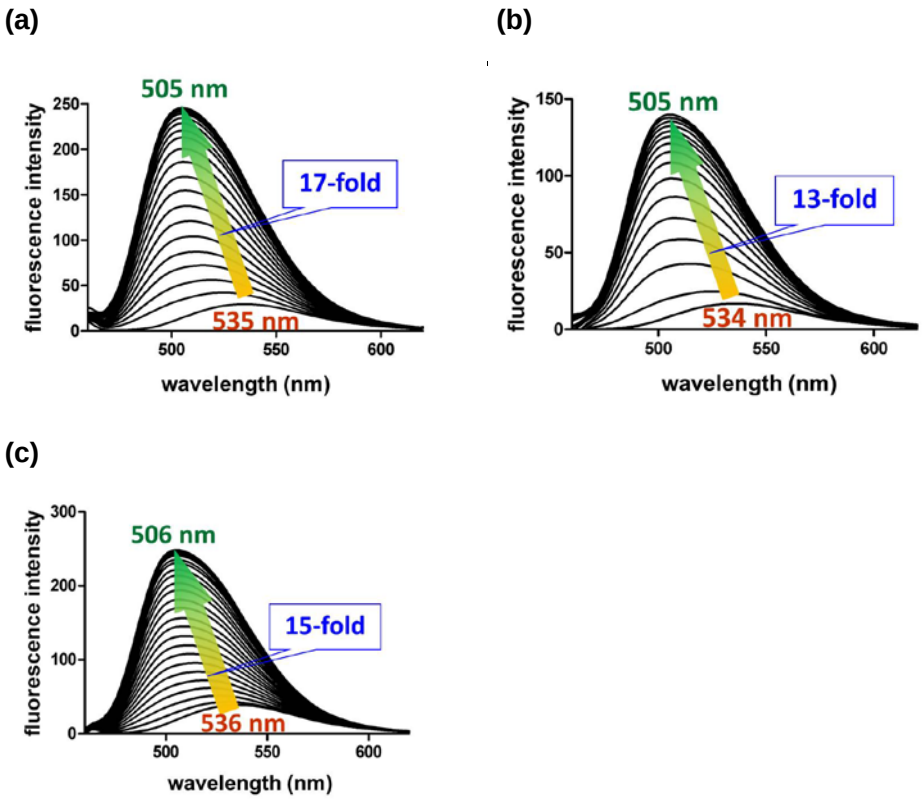



**Figure S1** Fluorescence spectral change of probes **1** (a), **2** (b), and **3** (c) upon addition of tag peptide at 25 °C in 50 mM HEPES buffer (pH 7.2, 100 mM NaCl), [probe] = 0.5 μM.

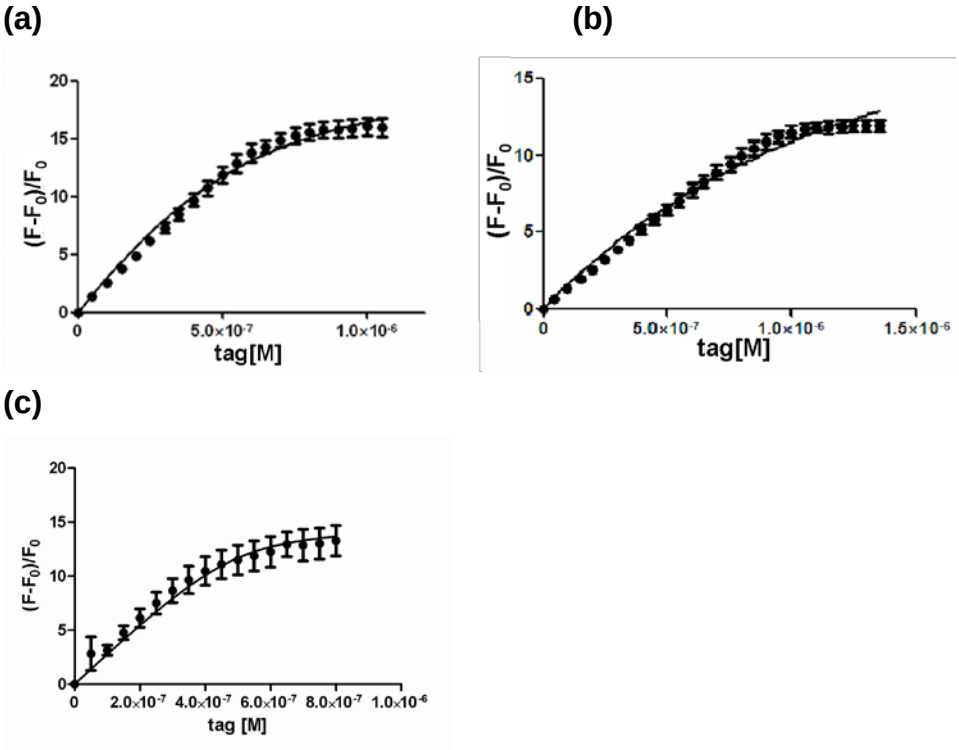



**Figure S2** Fluorescence titration curves of probes **1** (a), **2** (b), and **3** (c) with the tag peptide at 505, 505, and 506 nm, respectively. F and $F_0$ represent the fluorescence intensity at various concentrations of tag peptides and the initial fluorescence intensity, respectively.

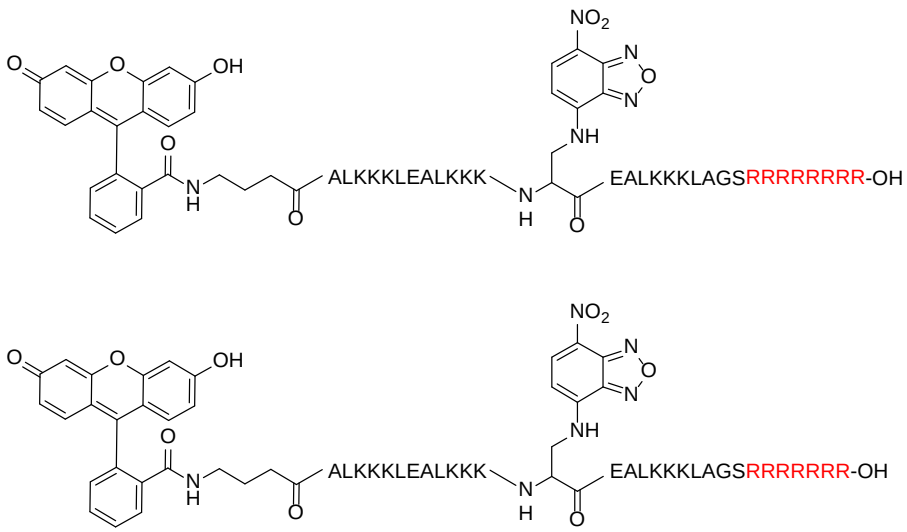



**Figure S3** Chemical structures of probes **4** (upper) and **5** (lower).

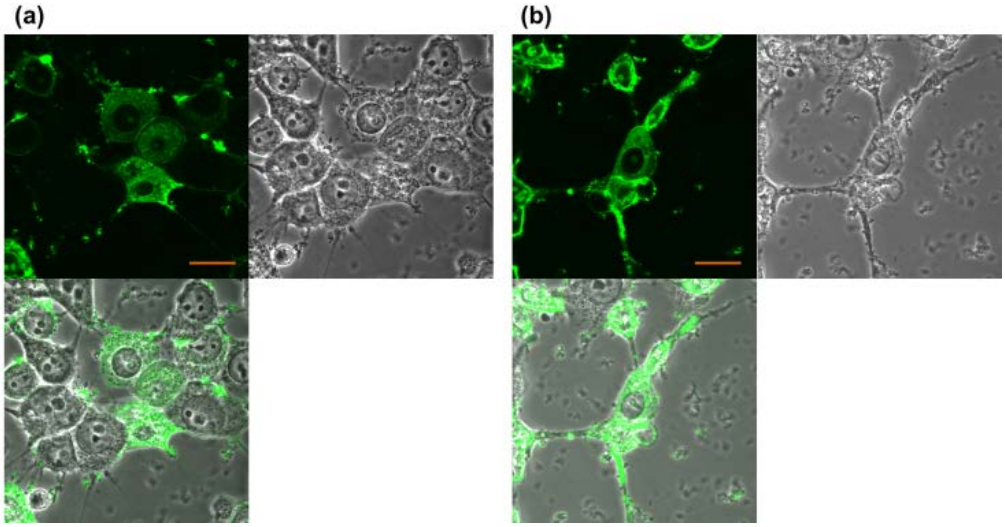



**Figure S4** Evaluation of cell permeability of probes **4** with R8 (a) and **5** with R7 (b). The probe concentration was 5 μM. For fluorescent imaging, a confocal laser-scanning microscope, FV10i (Olympus) equipped with a 40× objective lens, was utilized.

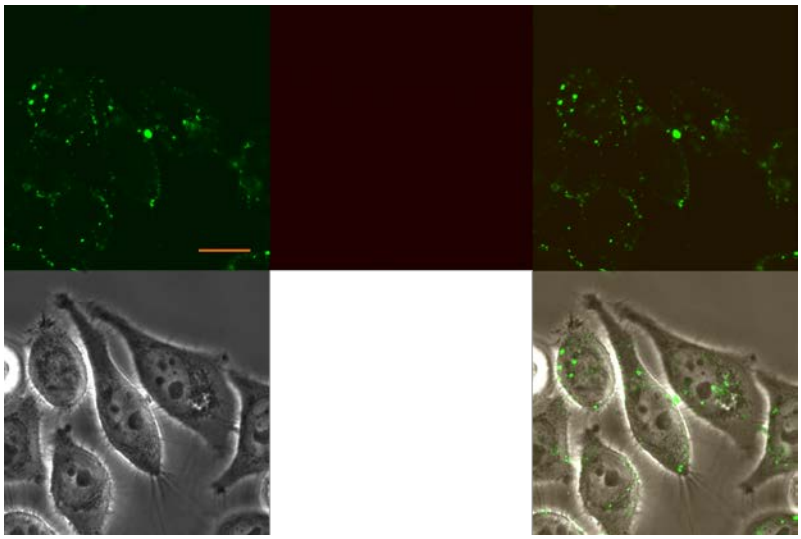


**Figure S5** Observation of cell penetration of probe **1** at 1 μM added to HeLa cells in the absence of target protein fused with mKO. Panels show the NBD signal (upper left), the mKO signal (upper middle), the merge of NBD and mKO signals (upper right), the DIC image (lower left), and the merge of NBD, mKO, and DIC (lower right). The scale bar indicates 20 μm. For fluorescent imaging, a confocal laser-scanning microscope, FV10i (Olympus) equipped with a 40× objective lens, was utilized.

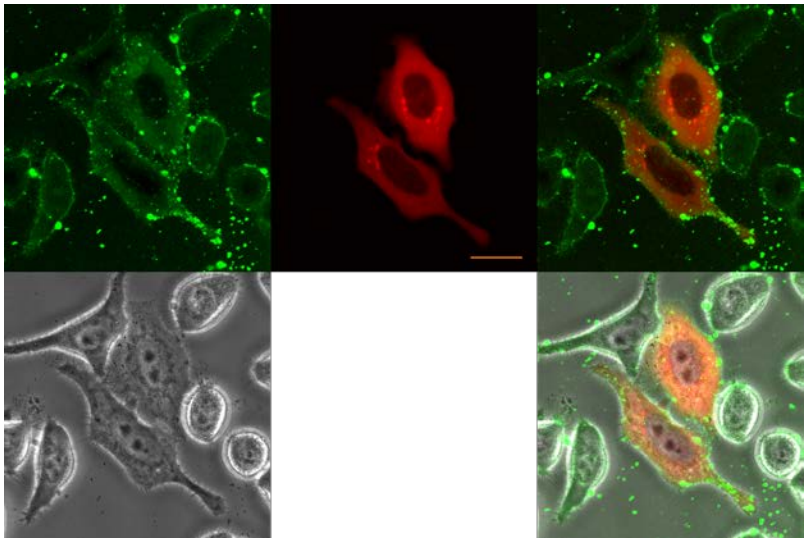


**Figure S6** Observation of cell penetration of probe **1** at 1 μM added to HeLa cells expressing PKCδ-mKO-tag after treatment by 100 μM 1-pyrenebutyrate. Panels show the NBD signal (upper left), the mKO signal (upper middle), the merge of NBD and mKO signals (upper right), the DIC image (lower left), and the merge of NBD, mKO, and DIC (lower right). The scale bar indicates 20 μm. For fluorescent imaging, a confocal laser-scanning microscope, FV10i (Olympus) equipped with a 40× objective lens, was utilized.

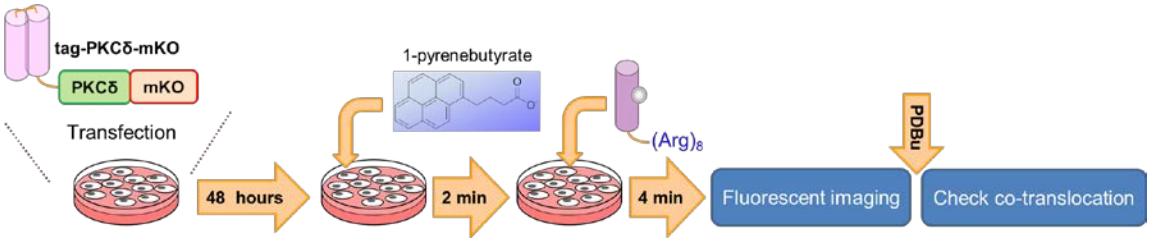



**Figure S7** Experimental scheme for co-localization detection between probe **1** and PKCδ-mKO-tag. Probe **1** was added after the treatment with 1-pyrenebutyrate.