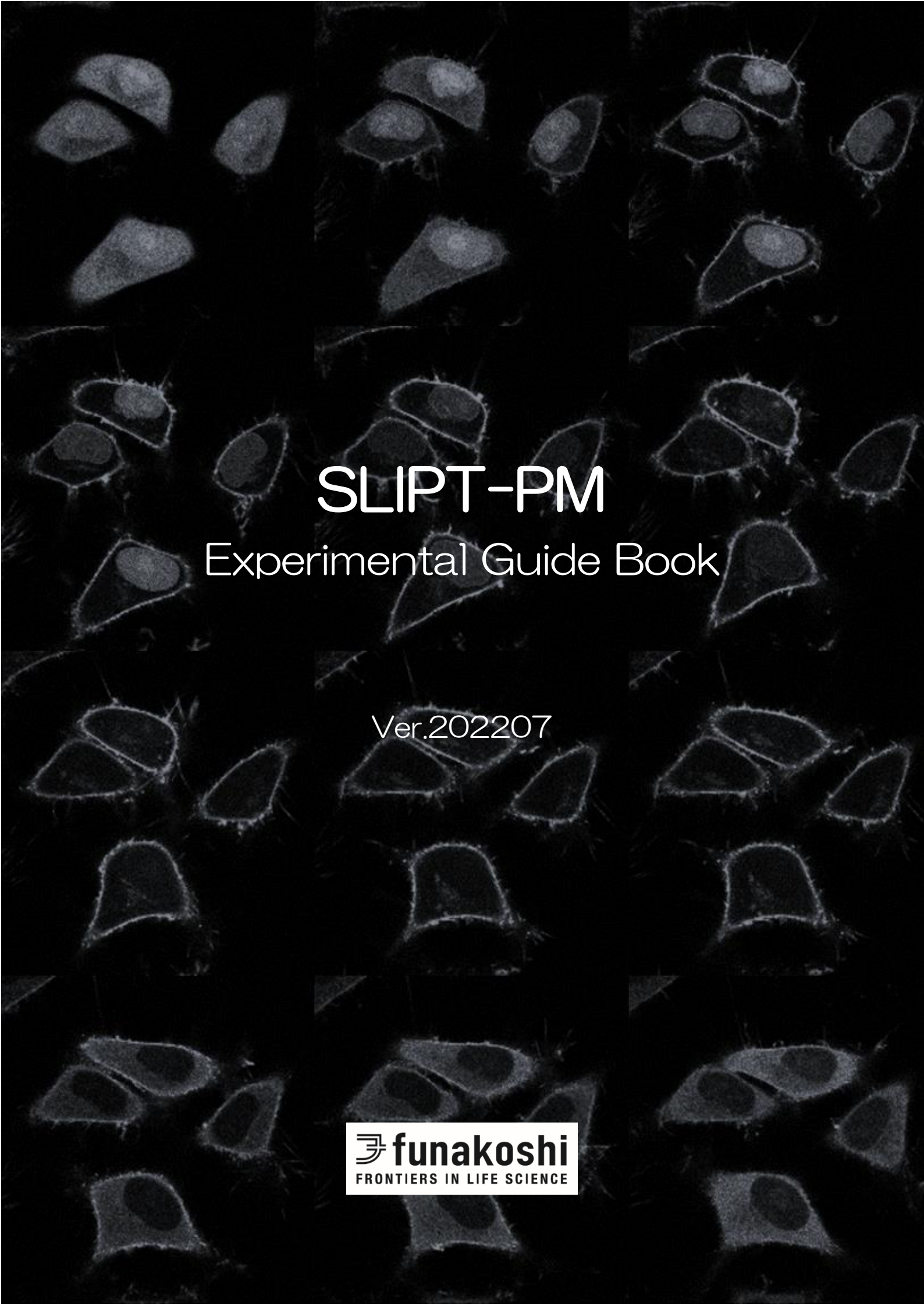


SLIPT-PM

Experimental Guide Book

Ver.202207

funakoshi
FRONTIERS IN LIFE SCIENCE

Index

1. What is SLIPT?

Self-localizing (SL) **l**igand-**i**nduced **p**rotein **t**ranslocation (SLIPT), originally developed by Dr. Shinya Tsukiji, Nagoya Institute of Technology, is a novel technology for controlling intracellular localization of proteins by small compounds. The SLIPT platform is a versatile single protein component system and can be applied to various proteins. SLIPT platform requires two primary components. One is a chemical reagent called **self-localizing (SL) ligand,** and another is a genetically engineered **tag-protein** that selectively binds to the SL ligand. SL ligand has a specific ligand for the tag-protein conjugated to an organelle-selective accumulation motif via a flexible linker. Until now, Dr. Tsukiji's group has successfully developed plasma membrane (PM), nucleus, endoplasmic reticulum (ER) membrane, Golgi apparatus membrane, and cytoskeleton-specific SL ligands. The **Protein of interest (POI)** fused to tag-protein can be translocated to the target organelle directionally by each organelle-selective SL ligand.

Among some tag-protein and SL ligand pairs, the most validated are ***E*.coli dihydrofolate reductase (eDHFR)** and its specific ligand **trimethoprim (TMP)** pair. TMP was developed initially as an antibacterial drug for *E.coli* and shows nM order of Kd value to eDHFR. On the other hand, TMP has a very low affinity to mammalian DHFR enzymes and little effects on mammalian cells. According to these properties, the eDHFR/TMP pair is considered a biorthogonal pair used in various cell biology fields.

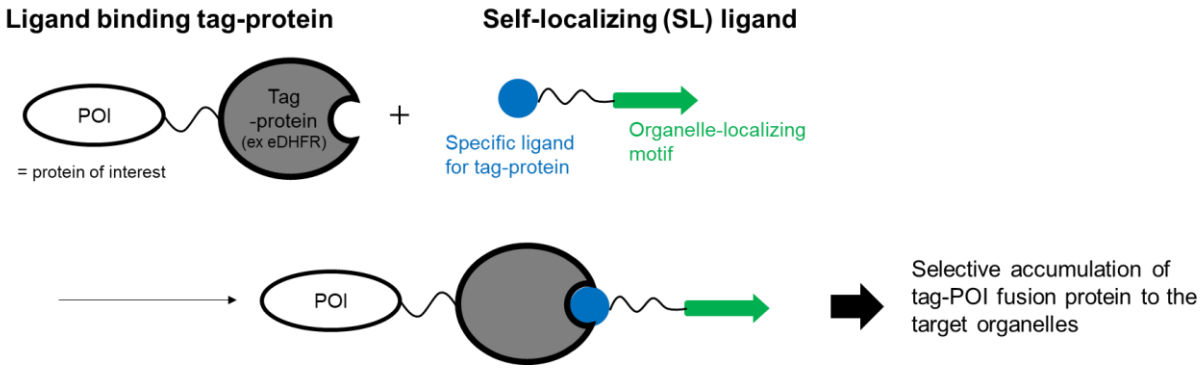


Figure 1.1 Concept of SLIPT platform

2. What is SLIPT-PM reagent?

SLIPT-PM reagent

Our **SLIPT-PM** reagent is a plasma membrane (PM)-selective SL ligand (original compound name; m^{D}cTMP, see Ref.2 and Ref.3) and used with a genetically engineered eDHFR mutant called ***^{iK6}*DHFR**. ^{iK6}DHFR-fused POI will rapidly translocate to the PM by SLIPT-PM reagent and stably tether on the inner leaflet of the PM. The PM-localized ^{iK6}DHFR-fused POI by SLIPT-PM reagent will quickly release to the cytosol by adding **Free-TMP**, which is provided as a kit component. PM-cytosolic shuttling of ^{iK6}DHFR-fused POI can be reversibly controlled several times by SLIPT-PM and Free-TMP (Fig 2-1).

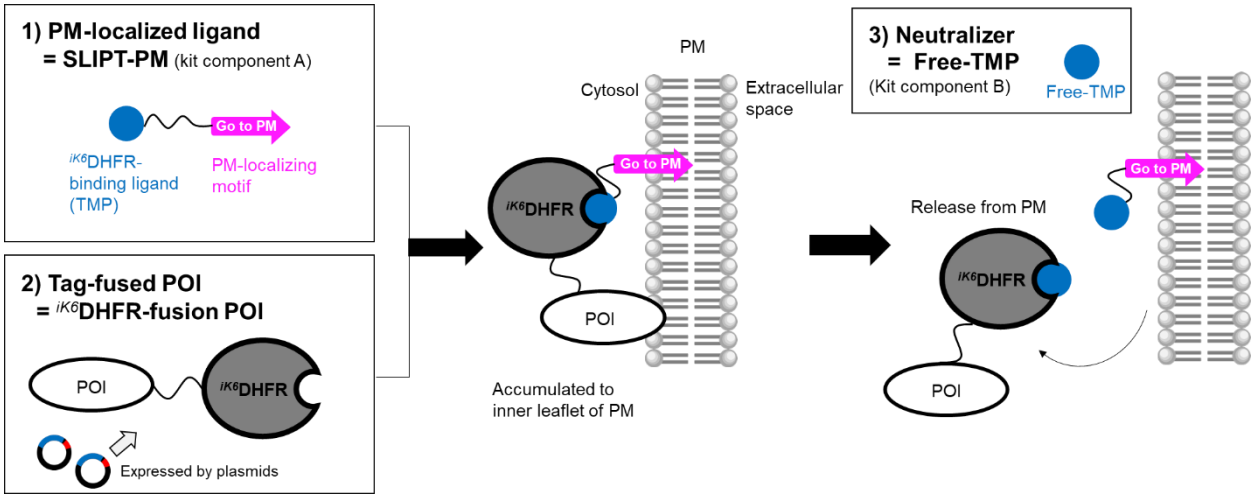


Figure 2-1 Overview of translocation of ^{iK6}DHFR-fused POI by SLIPT-PM and Free-TMP

Molecular structure of SLIPT-PM

SLIPT-PM has a TMP conjugated with *N*-myristoyl ^{D}Cys via a flexible PEG linker (Fig. 2-2 left). As *N*-myristoyl ^{D}Cys is a typical *S*-palmitoylation motif, SLIPT-PM is quickly *S*-palmitoylated by endogenous *S*-palmitoylation enzymes in the cells. *S*-palmitoylation is a reversible, enzymatic posttranslational modification of proteins in which the fatty acid chain, mainly palmitate, is attached to a thiol group of cysteine residue. Generally, *S*-palmitoylation of protein increases affinity to PM and Golgi apparatus membranes. Similar to *S*-palmitoylated proteins, *S*-palmitoylated SLIPT-PM (herein refer to **SLIPT-PM(Palm)**, Fig. 2-2 right) converted by endogenous *S*-palmitoylation enzymes in the cell is also accumulated to PM and Golgi apparatus and shows PM-Golgi apparatus shuttling by *S*-palmitoylation enzymes and depalmitoylation enzymes. One important point is that SLIPT-PM(Palm) localizes in both PM and Golgi apparatus membrane. When wild type eDHFR (eDHFR^{WT}) is fused to POI as a SLIPT-PM pair, eDHFR^{WT}-fused POI is translocated to PM and Golgi apparatus membranes by SLIPT-PM addition. **For PM-selective translocation of POI, genetically engineered eDHFR mutant ^{iK6}DHFR is required.** Detailed information on ^{iK6}DHFR is described in the next section.

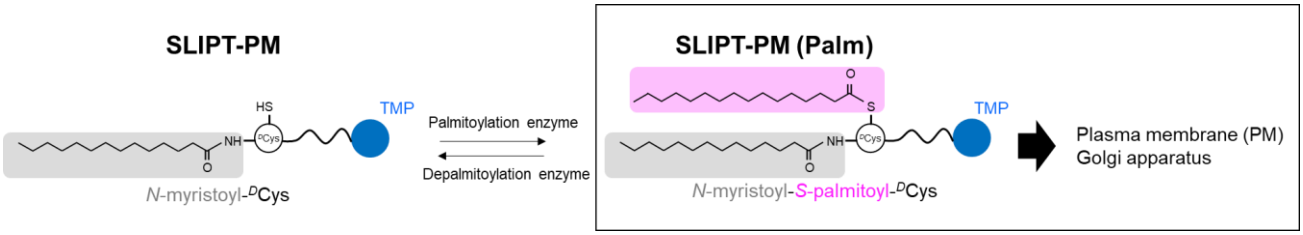


Figure 2-2 Molecular structure of SLIPT-PM and localization specificity

<u>^{iK6}DHFR, a SLIPT-PM specific eDHFR mutant</u>

As mentioned in the previous section, SLIPT-PM is incorporated into the cell and converted to SLIPT-PM(Palm) by *S*-palmitoylation enzymes, and subsequently, SLIPT-PM(Palm) is accumulated into both PM and Golgi apparatus. To establish PM-selective accumulation of eDHFR-fused POI, Dr. Tsukiji and co-workers recently developed ^{iK6}DHFR, an eDHFR mutant bearing hexa-lysine loop sequence (K6 loop) inserted into the internal region of eDHFR. The polycationic K6 loop of ^{iK6}DHFR increases the binding affinity of the eDHFR/SLIPT-PM(Palm) complex to the inner leaflet of the PM in which anionic lipids including phosphatidylserine etc. are enriched (Fig. 2-3). This electric interaction between ^{iK6}DHFR and the inner leaflet of the PM changes a balance of PM-Golgi apparatus shuttling of the complex of ^{iK6}DHFR and SLIPT-PM(Palm) and induces PM-selective translocation of ^{iK6}DHFR by SLIPT-PM (Fig. 2-4). Furthermore, ^{iK6}DHFR can be fused to both N- and C-terminal of POI with SLIPT-PM-induced PM-selective translocation activity (Fig. 2-5).

*DNA and amino acid sequences of ^{iK6}DHFR are referred in Chapter 8.

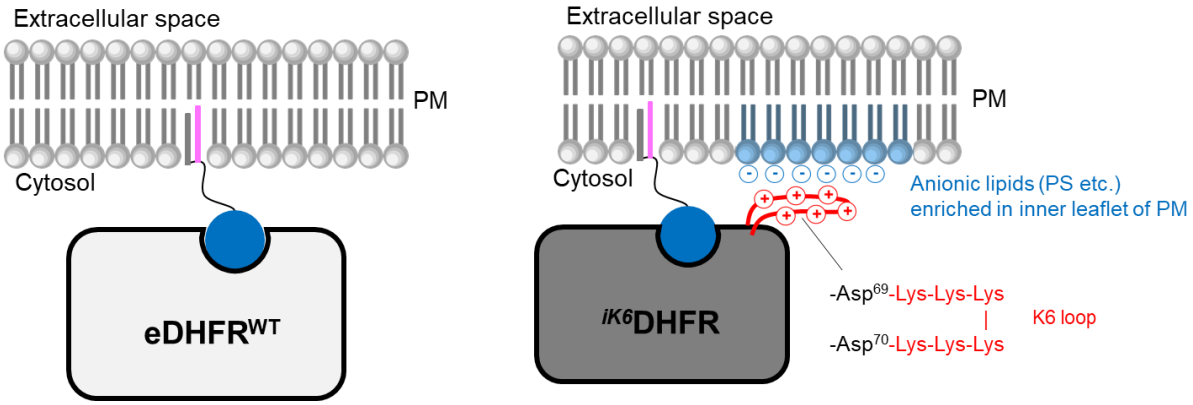


Figure 2-3 Comparison between eDHFR^{WT} and ^{iK6}DHFR

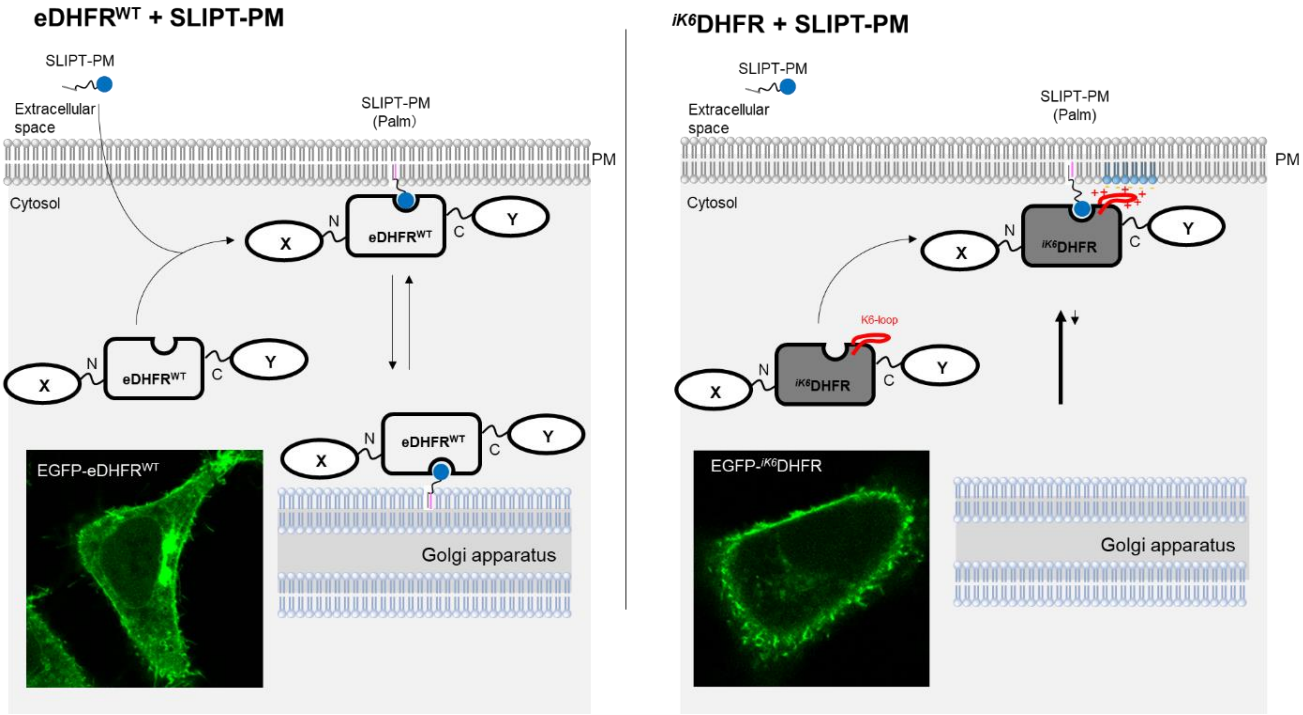


Figure 2-4 Localization difference between eDHFR^{WT} and ^{iK6}DHFR

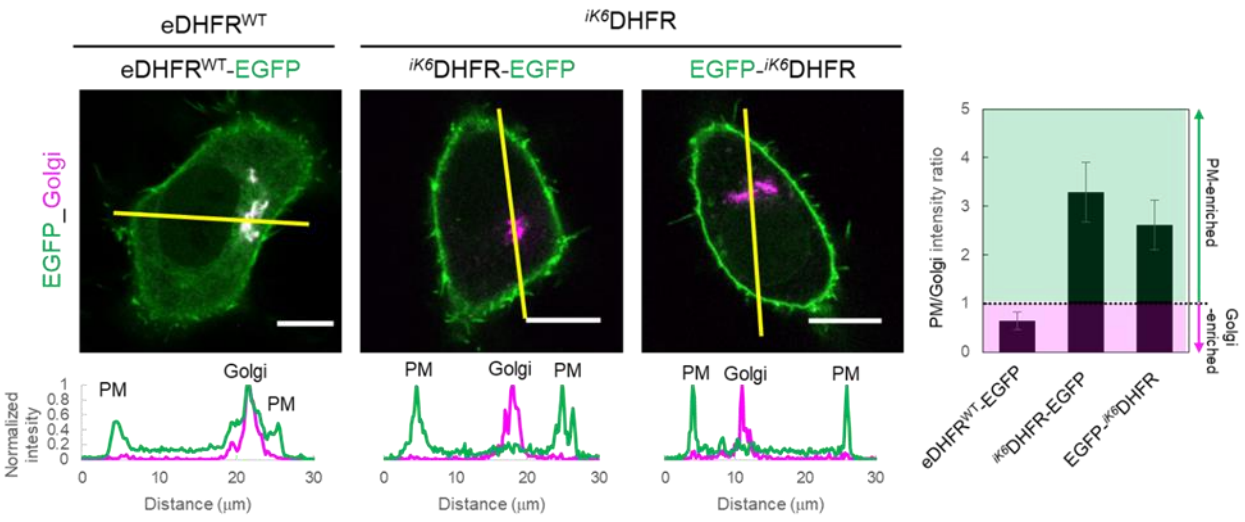


Figure 2-5 Quantitative analysis of localization specificity of eDHFR^{WT} and ^{iK6}DHFR

Superiority of SLIPT-PM on signal transduction researches

Analyses of signal transduction are commonly based on receptor stimulation by any specific extracellular ligands (Fig. 2-6 left). However, extracellular stimulation causes simultaneous activation of several pathways and shows the total cellular output from several signal transduction. Crosstalk of several signal pathways also complicates analyses of signal transduction. To overcome the problem, researchers developed novel research tools, but the versatile tools for specific activation of the signal pathway of interest are highly limited. The combination of SLIPT-PM and ^{iK6}DHFR-fused POI overcomes previous problems in signal transduction research.

If POI can express its functions on the PM, PM-recruitment of ^{iK6}DHFR-fused POI by SLIPT-PM activates the POI's function on the PM and induces activation of its downstream pathways. For example, an upstream factor of signal pathway A is expressed in cells as an ^{iK6}DHFR-fusion protein, translocated to the PM by SLIPT-PM and activated its following factors without any extracellular stimulation of the receptor (Figure 2-6 right). Dr. Tsukiji's group successfully validated some typical signaling pathways, including Ras signal, Rac signal, Gα signal, PI3K-Akt signal, etc.

<Experimentally validated signal pathways>

- cRaf-MEK-ERK pathway
- RasGEF-Ras-Raf-MEK-ERK pathway
- PKCδ-Raf-MEK-ERK pathway
- $G\alpha_q$-PLCβ-IP_3-Ca^{2+} pathway
- $G\alpha_s$-adenylate cyclase-cAMP pathway
- PI3K-PIP_2-PIP_3-Akt pathway
- RacGEF-Rac-actin pathway

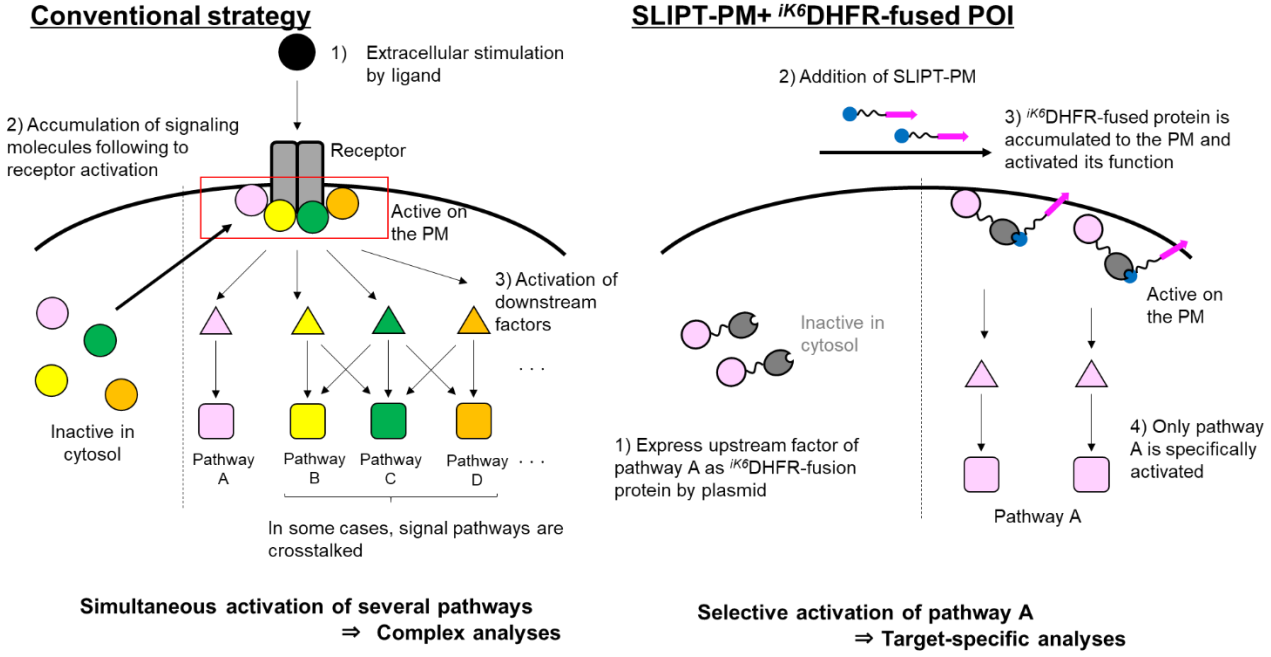


Figure 2-6 Superiority of SLIPT-PM/ ^{iK6}DHFR on signal transduction research

3. How to start SLIPT-PM experiments?

SLIPT-PM (catalog No. #FDV-0045) contains the following two components. This product does not include **^{iK6}DHFR-expression plasmids. ^{iK6}DHFR-expressing plasmids must be obtained from addgene or constructed from eDHFR^{WT}-expressing plasmids by yourself.**

SLIPT-PM kit components

A. SLIPT-PM 0.2 mg/vial

B. SLIPT supplement (Free-TMP) 5 mg/vial *Optionally used for PM release

Workflow of the establishment of SLIPT-PM

1. How to obtain ^{iK6}DHFR-expression plasmids?

^{iK6}DHFR-expression plasmids containing a multi-cloning site and EGFP gene are distributed by plasmid bank addgene (Table 3-1). You can also construct plasmids from eDHFR^{WT} gene by yourself, according to the reference paper (Ref. 3). Plasmids for control experiments mentioned in the guidebook are also distributed by addgene (Table 3-2).

Table 3-1 List of ^{iK6}DHFR-expression plasmids

Purpose	Plasmid name	Addgene ID
To fuse your POI to C-terminal of ^{iK6}DHFR (EGFP-^{iK6}DHFR-POI)	pEGFP-eDHFR(69K6)-MCS	172100
To fuse your POI to N-terminal of ^{iK6}DHFR (POI- ^{iK6}DHFR-EGFP)	pMCS-eDHFR(69K6)-EGFP	172101

* eDHFR(69K6) is another name of ^{iK6}DHFR

Table 3-2　List of plasmids for control experiments

Pathway	Construct name in the guidebook	addgene plasmid name	addgene ID
cRaf-MEK-ERK pathway	EGFP-*iK6*DHFR-cRaf	pPBpuro-EGFP-eDHFR(69K6)-cRaf	178849
	cRaf-mNeonGreen(mNG)-*iK6*DHFR	pPBpuro-cRaf-mNG-eDHFR(69K6)	172107
$G\alpha_q$ pathway	mNeonGreen(mNG)-*iK6*DHFR-$G\alpha_q$	pCAGGS-mNG-eDHFR(69K6)-G-alpha-q	172106
	mNeonGreen(mNG)-*iK6*DHFR-$G\alpha_q$ (L254A)	pCAGGS-mNG-eDHFR(69K6)-G-alpha-q(L254A)	178851
$G\alpha_s$ pathway	miRFP670-*iK6*DHFR-$G\alpha_s$	pPBpuro-miRFP670-eDHFR(69K6)-G-alpha-s	172105
	miRFP670-*iK6*DHFR	pCSIIpuro-miRFP670-eDHFR(69K6)	178852
PI3K-Akt pathway	mNeonGreen(mNG)-*iK6*DHFR-$p85_{iSH2}$	pPBpuro-mNG-eDHFR(69K6)-p85iSH2	172103
	mNeonGreen(mNG)-*iK6*DHFR	pCSIIpuro-mNG-eDHFR(69K6)	178853
RacGEF-Rac pathway	mNeonGreen(mNG)-*iK6*DHFR-$Tiam1_{DH-PH}$	pPBpuro-mNG-eDHFR(69K6)-Tiam1	172102
	mNeonGreen(mNG)-*iK6*DHFR	pCSIIpuro-mNG-eDHFR(69K6)	178853
Grb2/SOS1-Ras-Raf-MEK-ERK pathway	$Grb2_{mimic}$-iRFP713	pCAGGS-chGrb2/eDHFR(69K6)-iRFP713	178854
	*K6*DHFR-iRFP713	pCAGGS-eDHFR(69K6)-iRFP713	178855

Please find each application note in Chapter 6.

2) How to consider experimental design

To establish SLIPT-PM experiments, the design of ^{iK6}DHFR-fused POI proteins should be carefully considered in advance.

2-1) What kind of POI structure is expressed as ^{iK6}DHFR-fused protein

The structure of POI, for example, full length, any specific domains, and wild type or mutants for ^{iK6}DHFR-fused protein are essential for successfully establishing SLIPT-PM experiments. If the molecular size of full-length POI is very large, full-length POI may affect SLIPT-PM responses. In this case, any specific domains of POI for essentially expressing POI's function are desired. For reference, the longest length of validated POI is the full-length cRaf (~73 kDa, using in application data (1), p15). If POI has any potentially PM-binding capacity such as transmembrane domains, protein lipid modifications or PM protein-binding sites etc, POI may translocate to the PM independently of SLIPT-PM. In this case, PM-binding deficient mutants of POI should be considered.

2-2) How to design ^{iK6}DHFR-fused POI

As ^{iK6}DHFR can fuse to both N- and C-terminal of POI, you can select fusion sites of POI. Furthermore, the fusion of POI to both N- and C-terminal of ^{iK6}DHFR, such as X- ^{iK6}DHFR-Y, is also compatible. Some examples of domain substitution of PM-binding domain to ^{iK6}DHFR are successfully validated (Please see the application data (7), p27). Empirically optimize ^{iK6}DHFR-fused POI structure for your experiments.

<u>Consideration examples</u>

- Effect of N- or C-terminal structure on the POI function

  If the N- or C-terminal structure of POI is essential for its functions, a free terminal is desirable. For example, if the C-terminal of POI is important for its functions, N-terminal fusion of POI to ^{iK6}DHFR (EGFP-^{iK6}DHFR-POI) is desired.
- The necessity of linker sequence between POI and ^{iK6}DHFR

  In some cases, flexible linkers between POI and ^{iK6}DHFR increase POI's activity.
- Types and necessity of fluorescent proteins

  ^{iK6}DHFR-expression plasmids listed in Table 3-1 are EGFP expression plasmid-based. For your experiments, exchange EGFP to any fluorescent proteins of interest (fluorescent color, structure (monomer/dimer), etc.).

2-3) How to detect signaling output

All experimental data in this guidebook show the fluorescent imaging using fluorescent reporter proteins. On the other hand, a biochemical approach such as Western Blotting is compatible (see Ref.1). Carefully consider how to detect activation of POI and downstream signaling pathways of the POI in advance.

3) Confirm expression of the ^{iK6}DHFR fused POI protein

After plasmid transfection, the expression of ^{iK6}DHFR-fused POI should be confirmed by fluorescent imaging or Western Blotting. If the expression level is low, reconsideration of plasmid design is highly recommended. For example, check the compatibility of the plasmid promotor for your cells or the construction of ^{iK6}DHFR-fused POI.

4) Check localization of ^{iK6}DHFR-fused POI protein in steady-state

After confirming the expression of ^{iK6}DHFR-fused POI protein, the localization of ^{iK6}DHFR-fused POI protein in a steady-state should be confirmed. If ^{iK6}DHFR-fused POI protein localizes on the PM in a steady-state without SLIPT-PM, the addition of SLIPT-PM may not affect the localization of ^{iK6}DHFR fused POI. In these cases, ^{iK6}DHFR-fused POI has potentially membrane-binding activity, including transmembrane domains, protein lipid modifications such as palmitoylation and myristylation, and binding sites for membrane proteins. To dramatically translocate ^{iK6}DHFR-fused POI to the PM by the addition of SLIPT-PM, cytosolic localization of ^{iK6}DHFR fused POI in steady-state is desired. In some cases, ^{iK6}DHFR-fused POI may be accumulated into nucleus in steady-state but almost cases these ^{iK6}DHFR-fused POI can be translocated to the PM well by SLIPT-PM. If ^{iK6}DHFR-fused POI is strongly concentrated to the nucleus, nuclear export signal (NES) is one of the option to improve the localization of ^{iK6}DHFR-fused POI to the nucleus.

5) Optimization of SLIPT-PM condition

After confirmation of cytosolic localization of ^{iK6}DHFR-fused POI in steady-state, a response of localization of ^{iK6}DHFR-fused POI by SLIPT-PM addition is observed. Although the recommended concentration of SLIPT-PM is 1-10 μM, you must empirically optimize the concentration of SLIPT-PM.

IMPORTANT NOTICE

As serum components such as albumin may absorb SLIPT-PM, a serum-free medium is highly recommended. Dilute SLIPT-PM in serum-free medium or appropriate buffers.

6) Optimization of Free-TMP condition (OPTIONAL)

The Free-TMP releases ^{iK6}DHFR-fused POI from the PM to cytosol. You must empirically optimize the concentration of Free-TMP (5-10 eq. than SLIPT-PM) and its addition timing.

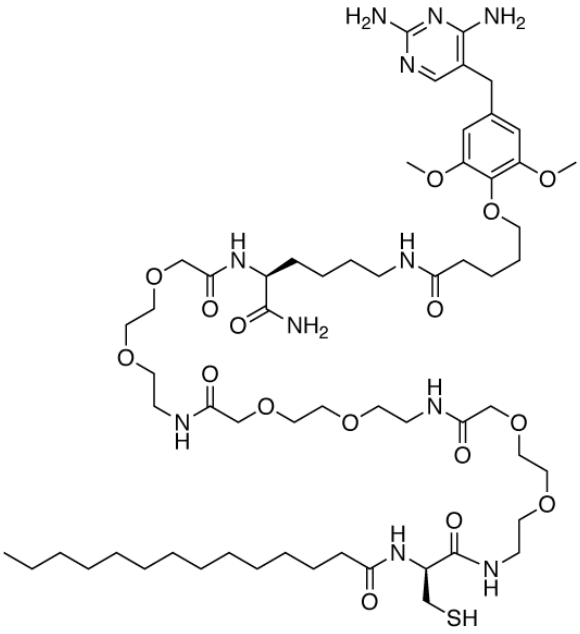
4. Product specification and protocol

● Product information

Catalog No. FDV-0045

Kit component and specification

	Component	Package	Molecular formulation	Molecular weight	Recommended solvent
A	SLIPT-PM (TFA salt)	0.2 mg	$C_{59}H_{101}N_{11}O_{16}S_1$ + CF_3COOH	1366.60 g/mol	DMSO
B	Free-TMP	5 mg	$C_{14}H_{18}N_4O_3$	290.32 g/mol	DMSO



SLIPT-PM

Storage condition:

Store at <-20°C

● How to prepare stock solution

A. SLIPT-PM

We recommend to prepare 5-10 mM in DMSO (0.2 mg/16-32 μL) as stock solution

NOTE 1 SLIPT-PM is colorless and low visible syrup and adhered in the bottom of tube.

1) Add 16-32 μL of DMSO to vial A and solubilized well by pipetting

2) Make small aliquots to prevent freeze and thaw cycle and store aliquots at less than -20°C

NOTE 2 SLIPT-PM has a free thiol group (-SH) which is an essential unit for its PM-localization. Under oxidative conditions, SLIPT-PM may form disulfide bonds, causing dimerization and will lose PM-localization activity. Although the DMSO stock solution is stable at least for one month under -20°C, we recommend using the stock solution as soon as possible.

B. Free-TMP

We recommend preparing 100 mM in DMSO (5 mg/172 μL) as a stock solution

1) Add 172 μL of DMSO to vial B and vigorously stir

2) Make small aliquots to prevent freeze and thaw cycle and store aliquots at less than -20°C.

● Protocol example

1) Make 1-10 μM SLIPT-PM in **serum-free medium** (hereafter called SLIPT-PM dilution) from the DMSO stock solution of SLIPT-PM.

NOTE 1 Optimal concentration of SLIPT-PM is highly dependent on cell type and ^{iK6}DHFR -fused POI. Empirically optimize and determine the concentration of SLIPT-PM for your experiments.

NOTE 2 SLIPT-PM may be absorbed by serum proteins, dilute SLIPT-PM with serum-free media.

NOTE 3 Prepare SLIPT-PM dilution just before experiments and immediately add to cells within a few minutes.

2) Wash cells expressing ^{iK6}DHFR-fused POI several times and add the SLIPT-PM dilution prepared in step 1) above.

NOTE 4 Direct addition of DMSO stock solution of SLIPT-PM to cells cultured in serum-free media is also possible. In this case, pipette to mix and create a homogeneous cell culture reaction solution.

3) Observe localization response of ^{iK6}DHFR-fused POI by any assay methods such as imaging, Western Blot, etc.

4) (OPTIONAL) Add 5-10 eq. of Free-TMP to cells

5. Demonstration experiment

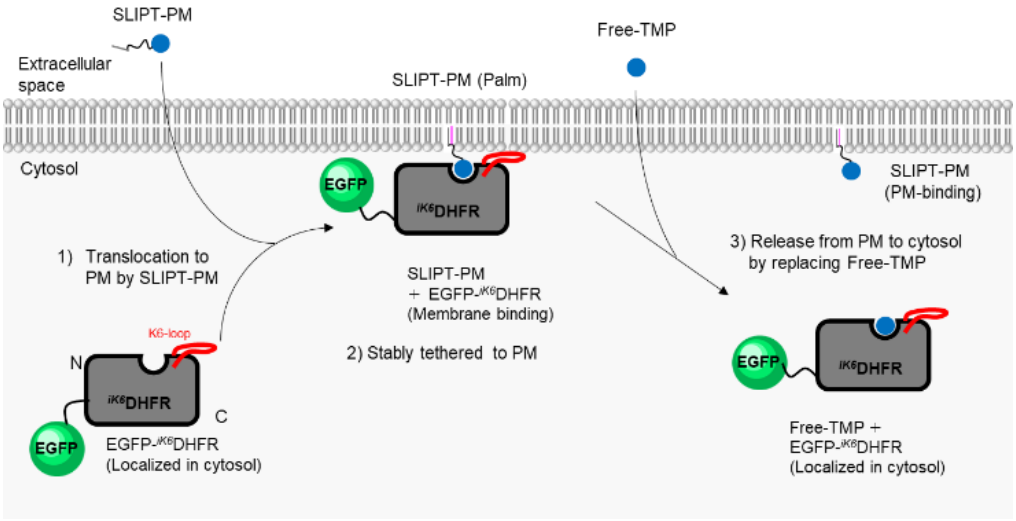
PM-translocation of EGFP-^{iK6}DHFR by SLIPT-PM and release from PM to cytosol by Free-TMP

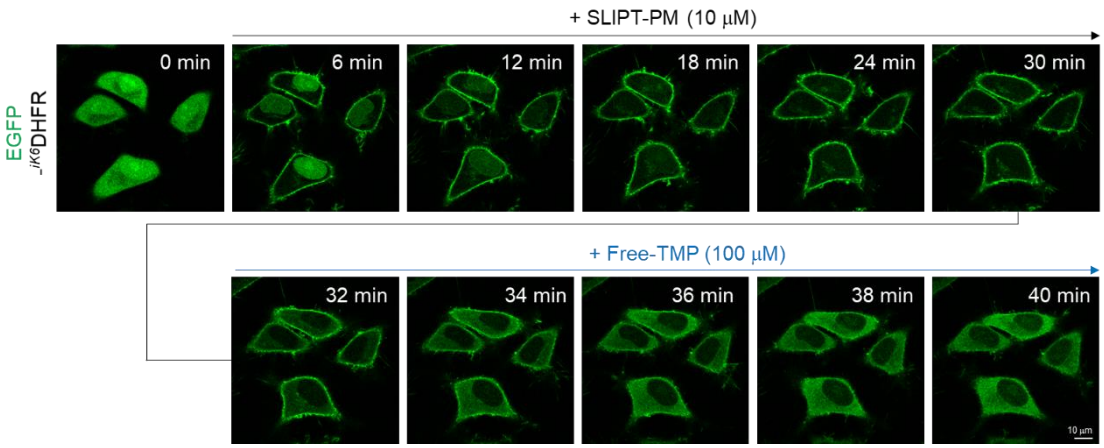
Experimental information

- Construction: EGFP-^{iK6}DHFR (Addgene ID: 172100)
- SLIPT-PM concentration: 10 μM (Addition at 0 min)
- Free-TMP concentration: 100 μM (Addition at 30 min)
- Cell line: HeLa cells
- Reaction medium: serum-free DMEM

Before the addition of SLIPT-PM, EGFP-^{iK6}DHFR is constantly localized in the cytosol in HeLa cells. Immediately after the addition of SLIPT-PM, EGFP-^{iK6}DHFR quickly translocated ($t_{1/2}$~1.5 min) to the PM and stably tethered in the PM, for at least several hours. By adding Free-TMP (30 min later), EGFP-^{iK6}DHFR gradually released from the PM to cytosol.

*A time-lapse movie is available on our website





6. Application data

(1) Activation of Raf-ERK pathway

Target signaling pathway: cRaf → MEK → ERK

Constructs in the experiment

- ^{iK6}DHFR-fused protein :EGFP-^{iK6}DHFR-cRaf^{*1} (addgene ID: 172107)

  ^{*1} In this experiment, cRaf indicates the full-length cRaf protein

- Reporter protein: Nucleus ERK activity sensor (^{ERK}KTR^{*2}-mKusabiraOrange (mKO))

  ^{*2} ^{ERK}KTR is kinase translocation reporter for ERK

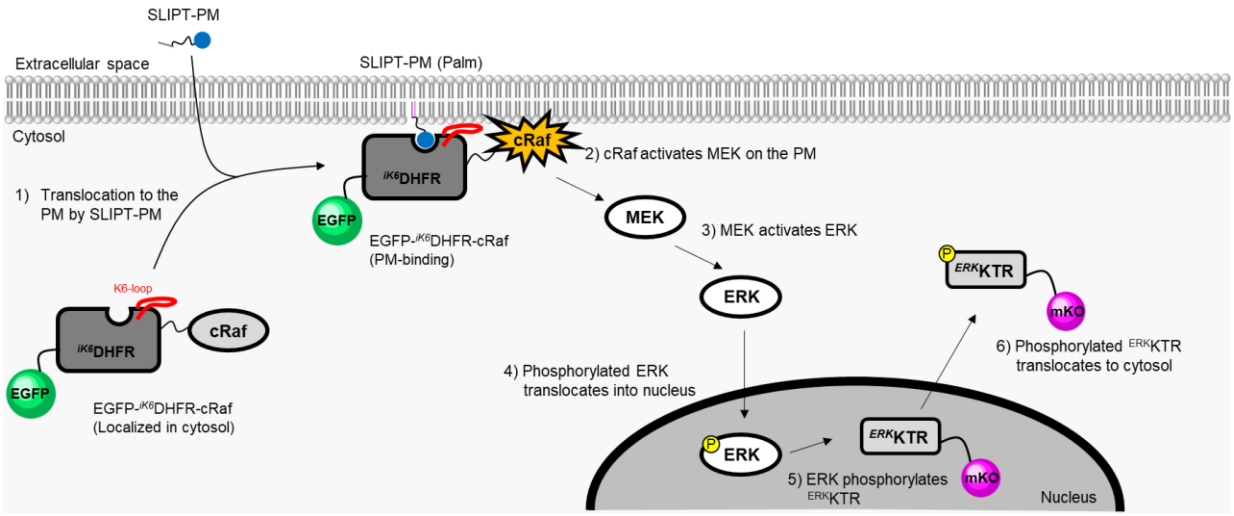
SLIPT-PM concentration: SLIPT-PM 10 μM

Cells: HeLa cells

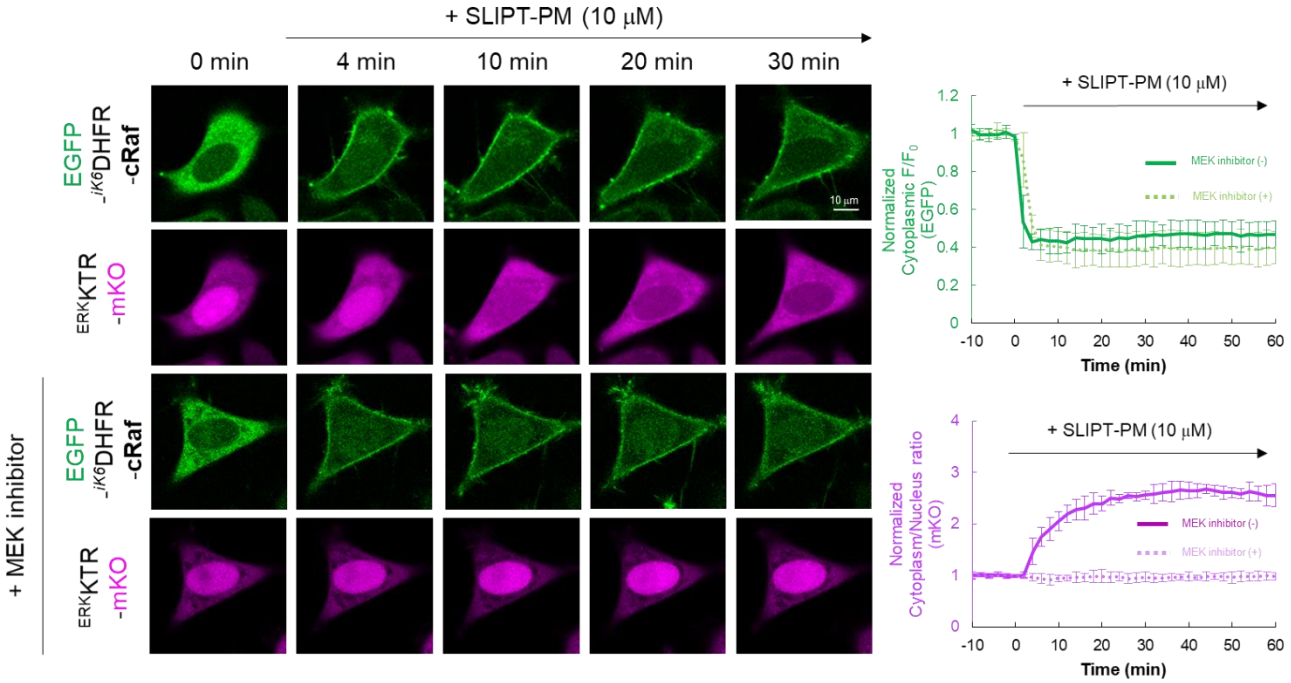
Reaction medium: serum-free DMEM

Experimental design:

cRaf, a downstream factor of Ras, can be activated by Ras on the PM and subsequently activate MEK-ERK pathway. Phosphorylated ERK is subsequently translocated into the nucleus and activates further downstream transcription factors. In this model experiment, ^{iK6}DHFR-fused cRaf protein (EGFP-^{iK6}DHFR-cRaf) is translocated to the PM by SLIPT-PM and promotes activation of MEK-ERK pathway on the PM without Ras activity. ERK activity is monitored by a nucleus ERK activity sensor (^{ERK}KTR-mKO).



Before the addition of SLIPT-PM, EGFP-^{iK6}DHFR-cRaf and ^{ERK}KTR-mKO were constantly localized in cytosol and mainly nucleus, respectively. By the addition of SLIPT-PM, at first, EGFP-^{iK6}DHFR-cRaf rapidly translocated to the PM ($t_{1/2}$~1 min) and subsequent elimination of nuclear ^{ERK}KTR-mKO to cytosol was observed. In the presence of MEK inhibitor, although EGFP-^{iK6}DHFR-cRaf translocated to the PM, ^{ERK}KTR-mKO localization was not changed. This result indicates SLIPT-PM promotes membrane-binding of EGFP-^{iK6}DHFR-cRaf and EGFP-^{iK6}DHFR-cRaf activates the MEK-ERK signaling pathway.



*A time-lapse movie is available on our website

(2) Activation of $G\alpha_q$ pathway

Target signaling pathway: $G\alpha_q$ → Phospholipase Cβ (PLCβ) → $PI(4,5)P_2$ (PIP_2) →
Diacylglycerol (DAG) + Inositol 1,4,5-trisphosphate (IP_3)→
IP_3 receptor type Ca^{2+} channel → Ca^{2+} release → Ca^{2+} oscillation

Constructs in the experiment

- ^{iK6}DHFR-fused protein: mNeonGreen (mNG)-^{iK6}DHFR-$G\alpha_q$[*1] (addgene ID: 172106)
  mNeonGreen (mNG)-^{iK6}DHFR-$G\alpha_q$(L254A)[*2] (addgene ID: 178851)

[*1] In this experiment, $G\alpha_q$ indicates Q209L/C9S/C10S mutant.
Q209L = constitutively active mutant
C9S/C10S = palmitoylation-deficient mutant, which has no spontaneous membrane-binding activity

[*2] $G\alpha_q$(L254A) indicates L254A/Q209L/C9S/C10S mutant.
L254A = PLCβ-binding deficient mutant which could not activate PLCβ

- Reporter protein: Ca^{2+} sensor (R-GECO1.0)

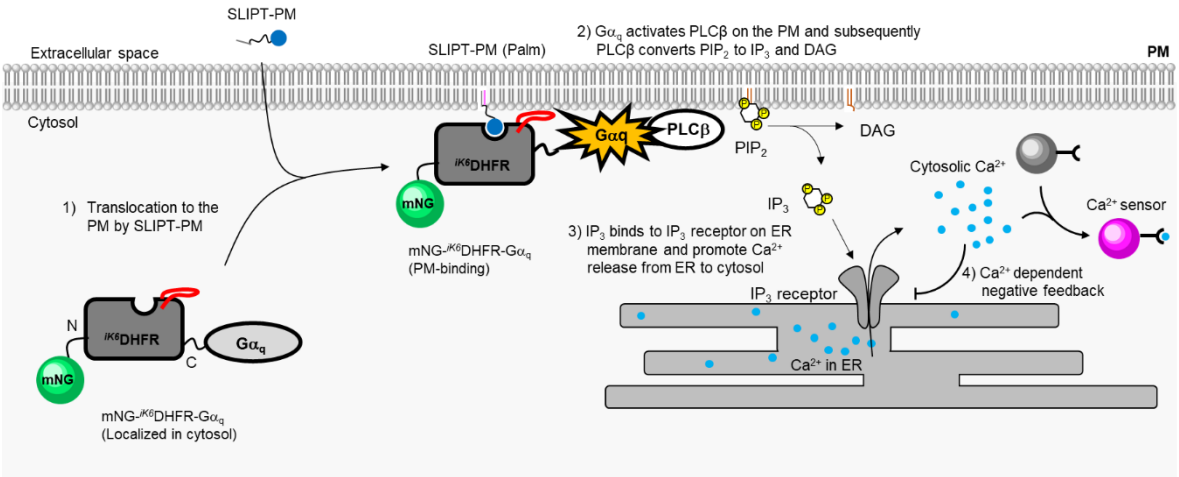
SLIPT-PM concentration: SLIPT-PM 5 μM

Cells: HeLa cells

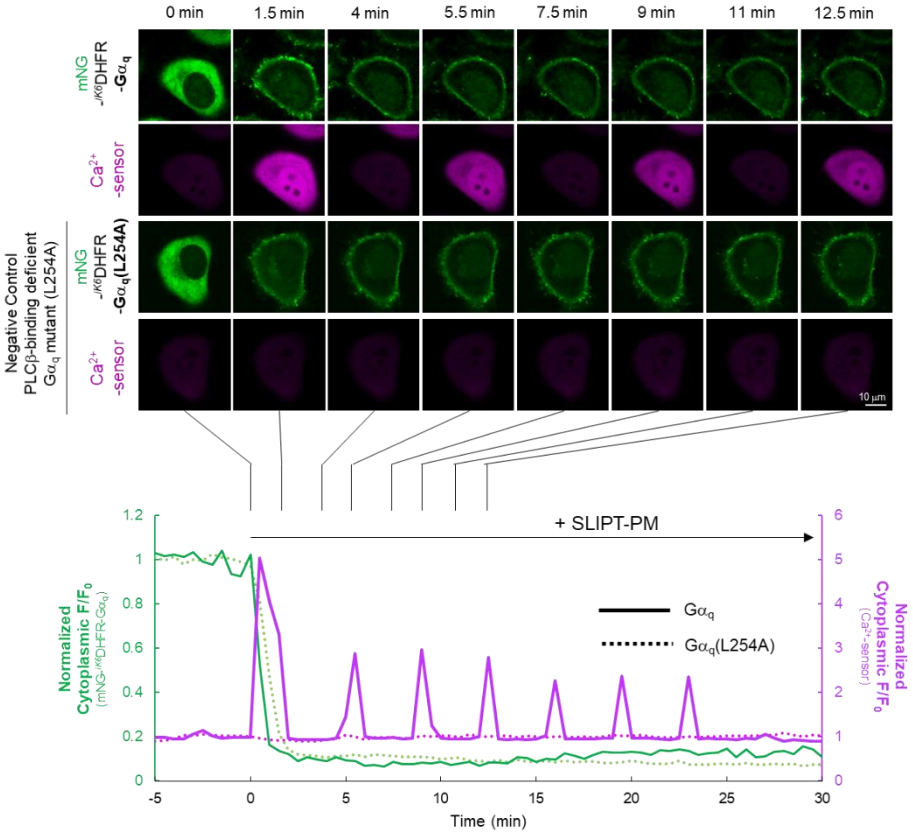
Reaction medium: HBSS containing 10 mM HEPES

Experimental design:

$G\alpha_q$, a subunit of GPCR-binding trimeric G proteins, is physiologically activated by GPCR-ligand stimulation and activates PLCβ. PLCβ subsequently converts PIP_2 to IP_3 and DAG on the PM. IP_3 is released from PM, binds to IP_3 receptor on ER and promotes Ca^{2+} release from ER to the cytosol. An increase of cytosolic Ca^{2+} also induces negative feedback of Ca^{2+} release. This reversible Ca^{2+} regulation shows Ca^{2+} oscillation. In this experimental model, ^{iK6}DHFR-fused $G\alpha_q$ (mNG-^{iK6}DHFR-$G\alpha_q$) is translocated to PM by SLIPT-PM and promotes interaction of $G\alpha_q$- PLCβ on the PM. Activated PLCβ by mNG-^{iK6}DHFR-$G\alpha_q$ on the PM subsequently promotes the conversion of PIP_2 and production of IP_3. IP_3 induces Ca^{2+} release and Ca^{2+} is detected by a genetically engineered Ca^{2+} fluorescent sensor (R-GECO).



Before the addition of SLIPT-PM, mNG-^{iK6}DHFR-Gα_q constantly localized in cytosol. By the addition of SLIPT-PM, mNG-^{iK6}DHFR-Gα_q rapidly translocated to the PM ($t_{1/2}$~1 min). After the PM recruitment of mNG-^{iK6}DHFR-Gα_q, dynamic Ca^{2+} oscillations were observed. In contrast, although mNG-^{iK6}DHFR-Gα_q(L254A) is also translocated to the PM by SLIPT-PM, no Ca^{2+} oscillations were detectable. This result indicates the PM recruitment of mNG-^{iK6}DHFR-Gα_q by SLIPT-PM can induce the Gα_q-downstream signaling pathway, including Ca^{2+} oscillations.



*A time-lapse movie is available on our website

(3) Activation of $G\alpha_s$ pathway

Target signaling pathway: $G\alpha_s$ → Adenylate cyclase (AC) → cAMP production

Constructs in the experiment

- ^{iK6}DHFR-fused protein: miRFP670-^{iK6}DHFR-$G\alpha_s$[*1] (addgene ID: 172105)
  miRFP670-^{iK6}DHFR (addgene ID: 178852)

[*1] In this experiment, $G\alpha_s$ indicates C3S mutant.

C3S = palmitoylation-deficient mutant, which has no spontaneous membrane-binding activity

- Reporter protein: Genetically engineered FRET-type cAMP sensor protein

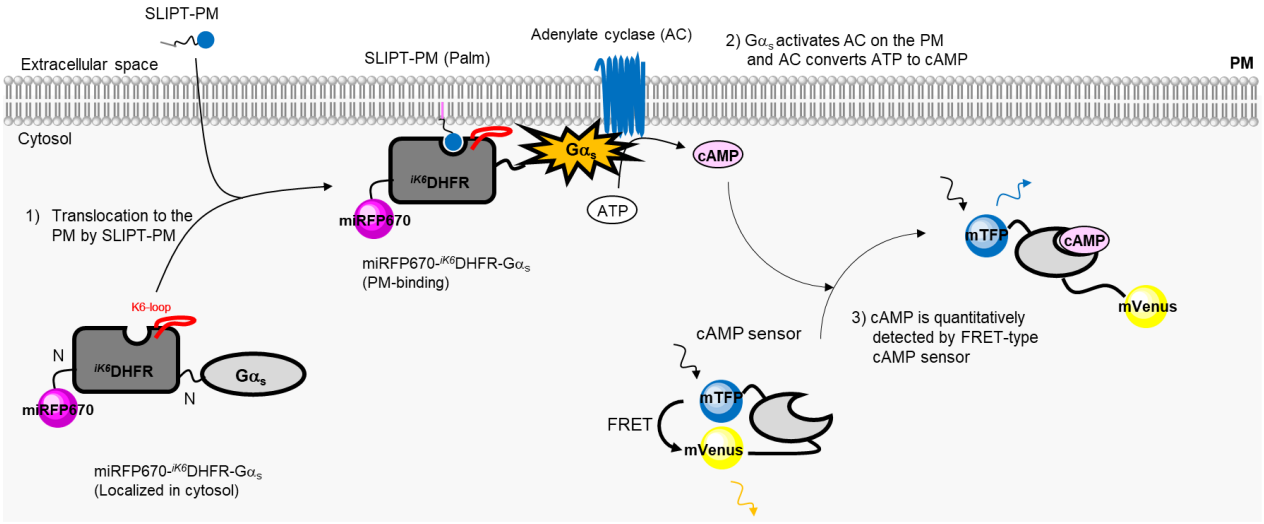
SLIPT-PM concentration: SLIPT-PM 10 μM

Cells: HeLa cells

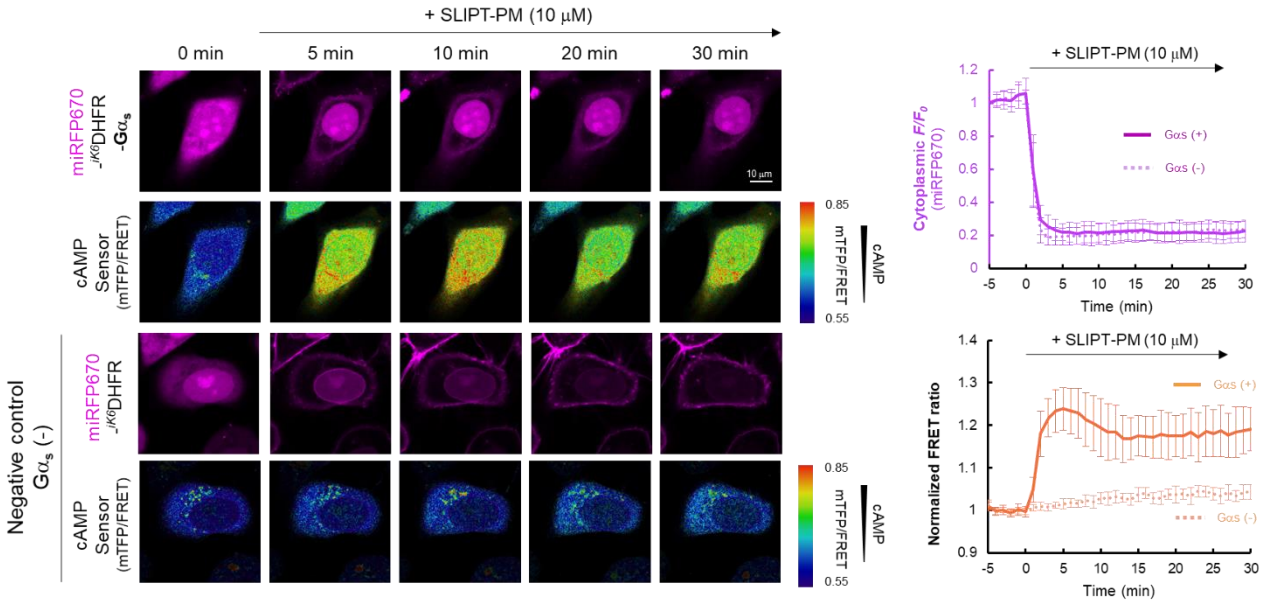
Reaction medium: serum-free DMEM

Experimental design:

$G\alpha_s$, a subunit of GPCR-binding trimeric G proteins, is physiologically activated by GPCR-ligand stimulation and activates AC. AC subsequently converts ATP to cAMP, a major second messenger for various signaling pathways, including PKA. In this experimental model, ^{iK6}DHFR-fused $G\alpha_s$ (miRFP670-^{iK6}DHFR-$G\alpha_s$) is translocated to the PM by SLIPT-PM and promotes interaction between ^{iK6}DHFR-fused $G\alpha_s$ and endogenous AC to induce cAMP production. The amount of cAMP change was observed by a genetically engineered FRET-type cAMP sensor protein.



Before the addition of SLIPT-PM, miRFP670-^{iK6}DHFR-Gα_s is constantly localized in cytosol. By the addition of SLIPT-PM (10 μM), miRFP670-^{iK6}DHFR-Gα_s rapidly translocated to the PM ($t_{1/2}$~1 min). After PM recruitment of miRFP670-^{iK6}DHFR-Gα_s, the FRET ratio (mTFP/mVenus) of cAMP sensor was dramatically changed, indicating a rapid increase of cAMP concentration. In contrast, a Gα_s–deficient control, miRFP670-^{iK6}DHFR, could quickly translocate to the PM but no change of FRET ratio of the cAMP sensor. This result indicates PM recruitment of miRFP670-^{iK6}DHFR-Gα_s by SLIPT-PM induces the Gα_s-downstream signaling pathway, including cAMP production.



*A time-lapse movie is available on our website

(4) Activation of RasGEF (Tiam1) - Rac pathway

Target signaling pathway: RacGEF (Tiam1) → Rac → Arp2/3, PAK etc. → actin polymerization → Lamellipodium formation

Constructs in the experiment

- ^{iK6}DHFR-fused protein: mNeonGreen (mNG)-^{iK6}DHFR-Tiam1$_{DH\text{-}PH}$[*1] (addgene ID: 172102)
  mNG-^{iK6}DHFR (addgene ID: 178853)

[*1] In this experiment, Tiam1 indicates a catalytically active DH-PH domain (Tiam1$_{DH\text{-}PH}$) .

- Reporter protein: Genetically engineered actin sensor protein (Lifeact-mCherry)

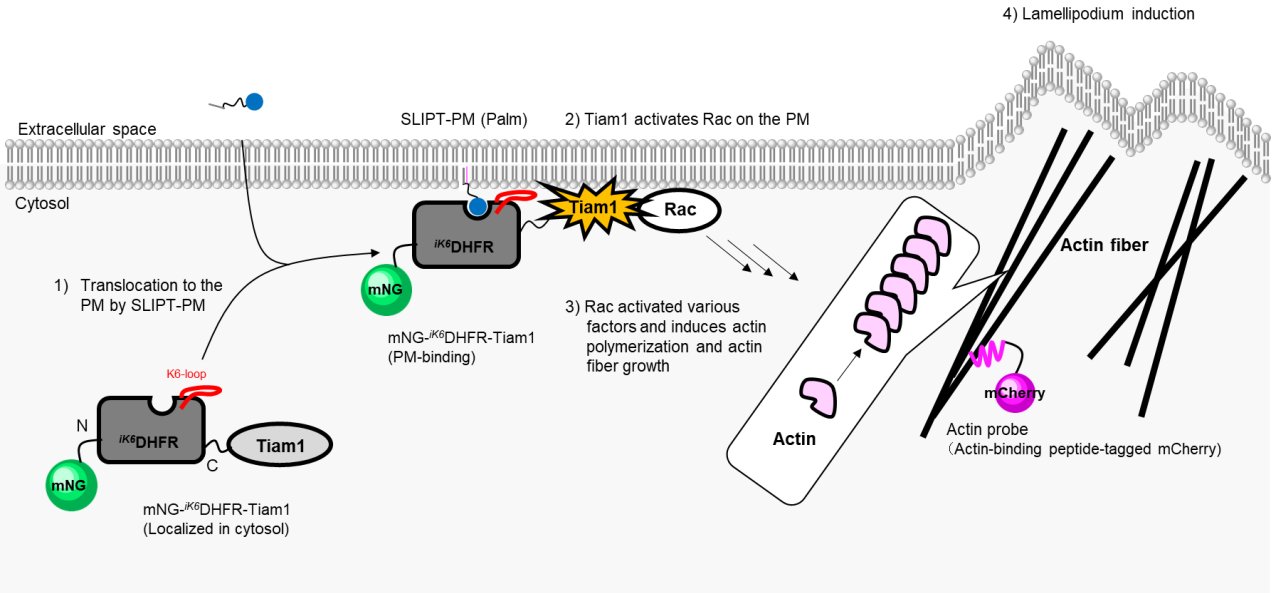
SLIPT-PM concentration: SLIPT-PM 5 μM

Cells: HeLa cells

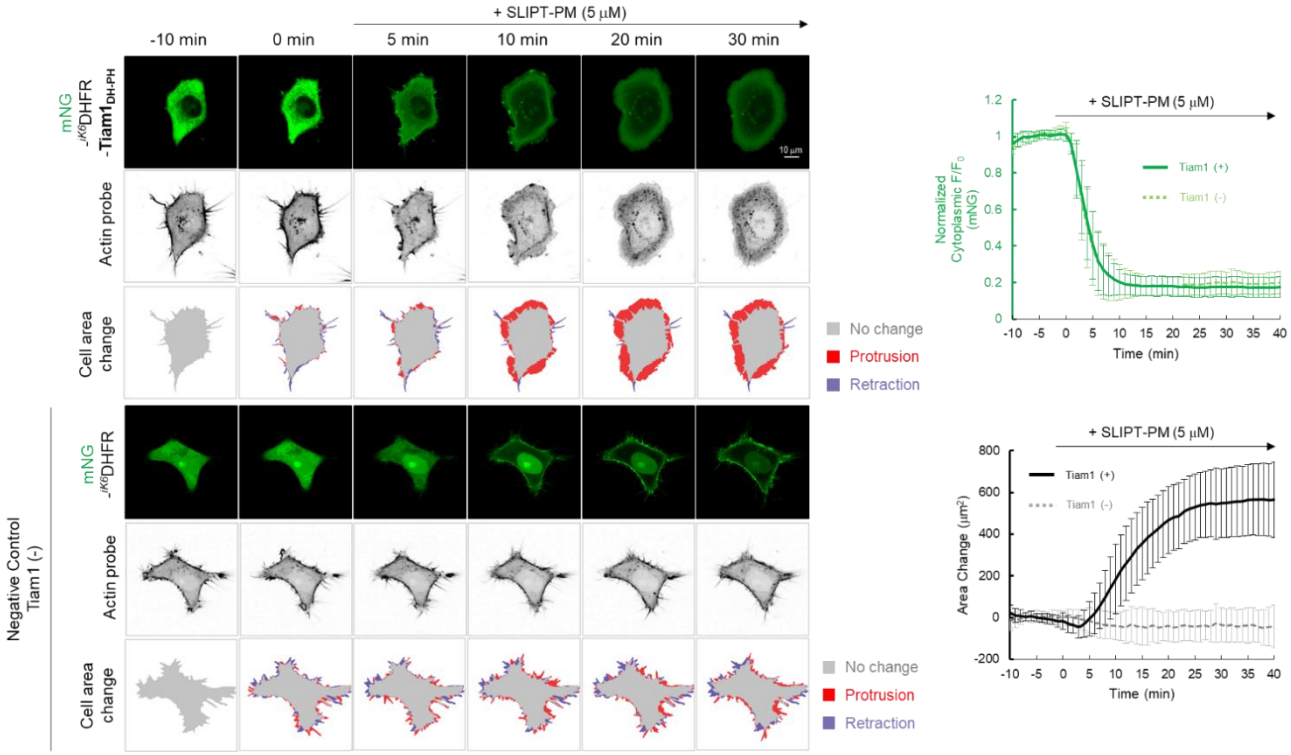
Reaction medium: serum-free DMEM

Experimental design:

Rac is a group of Rho family small GTP-binding proteins and Tiam1 is a member of guanine nucleotide exchange factor (GEF) for Rac (RacGEF). RacGEF is physiologically activated by upstream receptors and activates Rac on the PM. Subsequently, Rac activates various downstream factors, including Arp2/3 and PAK and promotes actin polymerization and reorganization of actin fibers. Actin reorganization induces cellular lamellipodium formation and dramatic cellular ruffling. In this experimental model, ^{iK6}DHFR-fused Tiam1 (mNG-^{iK6}DHFR-Tiam1$_{DH\text{-}PH}$) is translocated to the PM by SLIPT-PM and promotes interaction of mNG-^{iK6}DHFR-Tiam1$_{DH\text{-}PH}$ and endogenous Rac to induce Rac downstream actin polymerized signaling. Reorganization of actin fibers is monitored by a genetically encoded actin sensor protein and cell area is calculated from cellular morphology.



Before the addition of SLIPT-PM, mNG-^{iK6}DHFR-Tiam1$_{DH-PH}$ constantly localized in cytosol. By the addition of SLIPT-PM (5 μM), mNG-^{iK6}DHFR-Tiam1$_{DH-PH}$ rapidly translocated to the PM ($t_{1/2}$~3 min). After the PM recruitment of mNG-^{iK6}DHFR-Tiam1$_{DH-PH}$, the actin structure dramatically changed from fillopodium to lamelipodium with area increase. In contrast, a Tiam1-deficient mutant mNG-^{iK6}DHFR could quickly translocate to the PM, but no change of cellular morphology. This result indicates the PM recruitment of mNG-^{iK6}DHFR- Tiam1$_{DH-PH}$ by SLIPT-PM can induce the Rac-downstream signaling pathway, including actin reorganization and lamellipodium formation.



*A time-lapse movie is available on our website

(5) Activation of PI3K-Akt pathway

Target signaling pathway: Phosphoinositide 3-kinase (PI3K) → PI(3,4,5)P_3 (PIP_3) →Akt

Constructs in the experiment

- ^{iK6}DHFR-fused protein: mNeonGreen (mNG) -^{iK6}DHFR-p85$_{iSH2}$[*1] (addgene ID: 172103)
  mNG-^{iK6}DHFR (addgene ID: 172103)

  [*1] In this experiment, p85 indicates iSH2 (inter-Src homology 2; p85$_{iSH2}$) domain which binds to endogenous p110 and shows PI3K activity.

- Reporter protein: mCherry-Akt$_{PH}$[*2]

  [*2] In this experiment, Akt indicates pleckstrin homology (PH; Akt$_{PH}$) domain which directly binds to PIP_3.

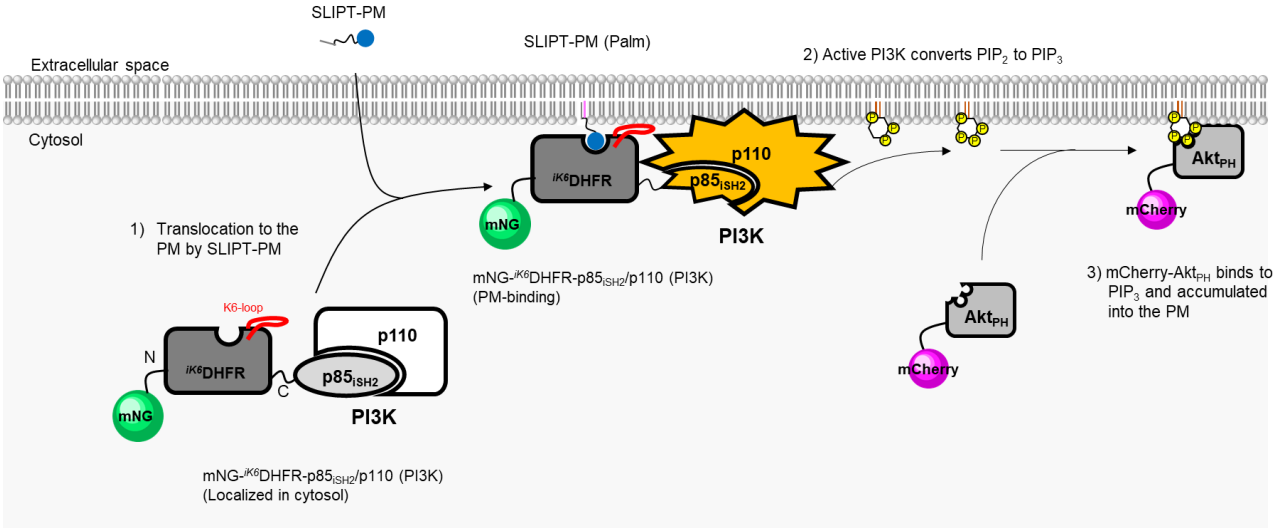
SLIPT-PM concentration: SLIPT-PM 10 μM

Cells: HeLa cells

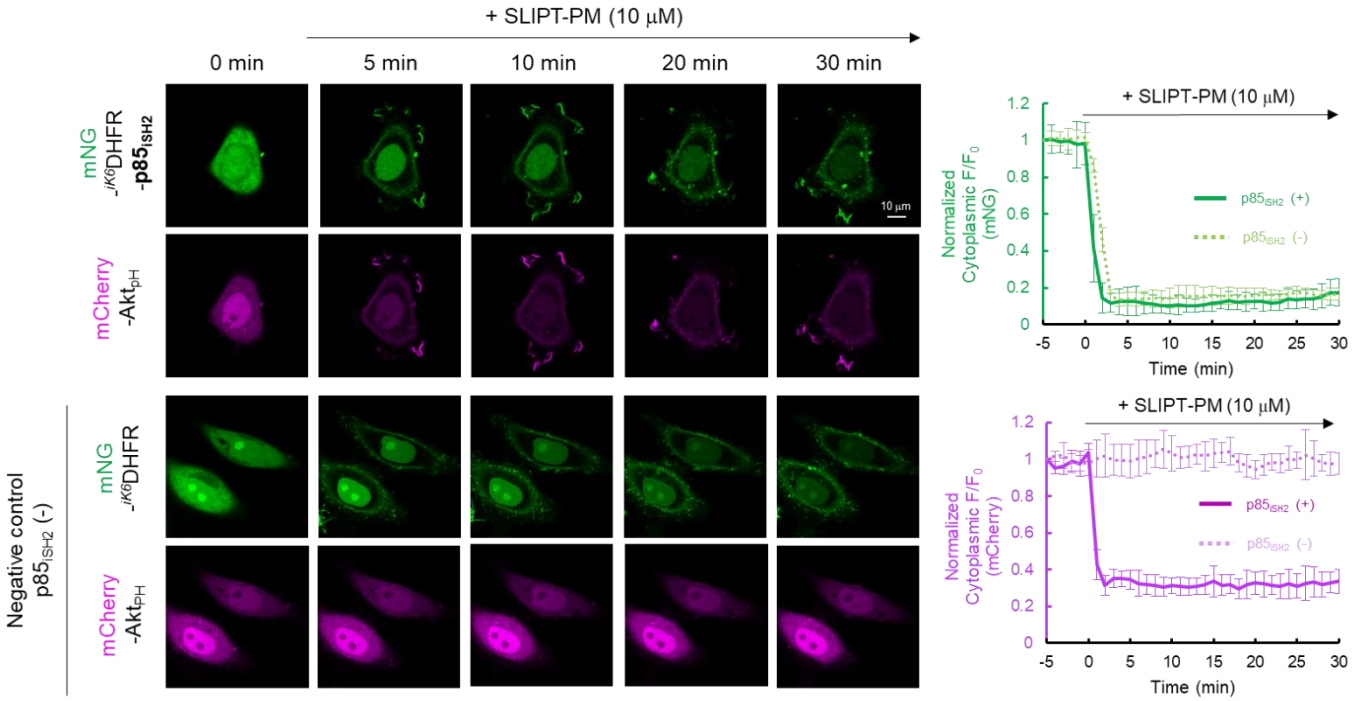
Reaction medium: serum-free DMEM

Experimental design:

PI3K, a complex of p85 and p110, is physiologically activated by various receptors with ligand stimulation and converts PIP_2 to PIP_3. Akt, a serine/threonine kinase, is subsequently activated by PIP_3 produced by PI3K and further contributes to various signaling pathways, including cell proliferation. In this experimental model, ^{iK6}DHFR-fused p85 (mNG-^{iK6}DHFR-p85$_{iSH2}$) bound with endogenous p110 is translocated to the PM by SLIPT-PM and promotes the production of PIP_3. The PIP_3 production is monitored by mCherry-fused Akt (mCherry-Akt$_{PH}$).



Before the addition of SLIPT-PM, both mNG-^{iK6}DHFR-p85$_{iSH2}$ and mCherry-Akt$_{PH}$ were constantly localized in cytosol. By the addition of SLIPT-PM (10 μM), mNG-^{iK6}DHFR-p85$_{iSH2}$ rapidly translocated to the PM ($t_{1/2}$~2 min). After the PM recruitment of mNG-^{iK6}DHFR-p85$_{iSH2}$, mCherry-Akt$_{PH}$ also rapidly translocated to the PM. In contrast, a p85$_{iSH2}$–deficient control, mNG-^{iK6}DHFR, could quickly translocate to the PM, but no change of localization of mCherry-Akt$_{PH}$. This result indicates the PM recruitment of mNG-^{iK6}DHFR- p85$_{iSH2}$/p110 complex by SLIPT-PM can induce the PI3K-downstream signaling pathway, including PIP_3 production.



*A time-lapse movie is available on our website

(6) Reversible Activation of Raf-ERK pathway

Target signaling pathway: cRaf → MEK → ERK

Constructs in the experiment

- ^{iK6}DHFR-fused protein : cRaf[*1]-mNeonGreen (mNG) -^{iK6}DHFR

  [*1] In this experiment, cRaf indicates the full-length cRaf protein

- Reporter protein: mCherry-ERK

SLIPT-PM concentration: 10 μM

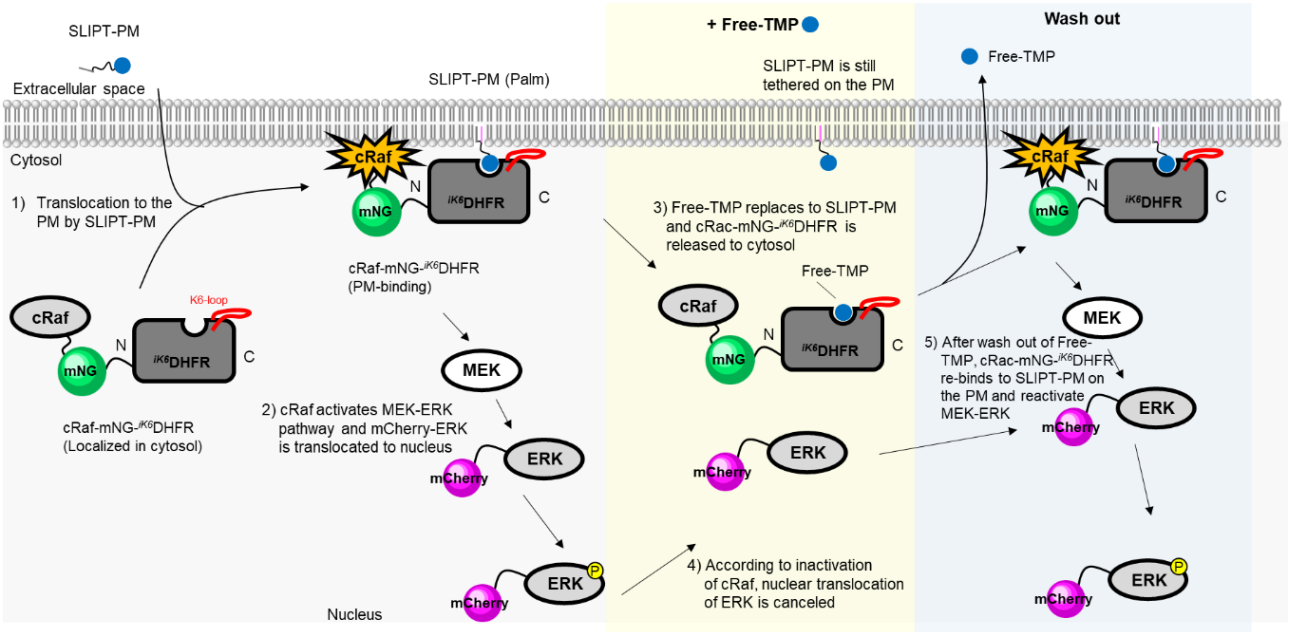
Free-TMP concentration: 50 μM

Cells: HeLa cells

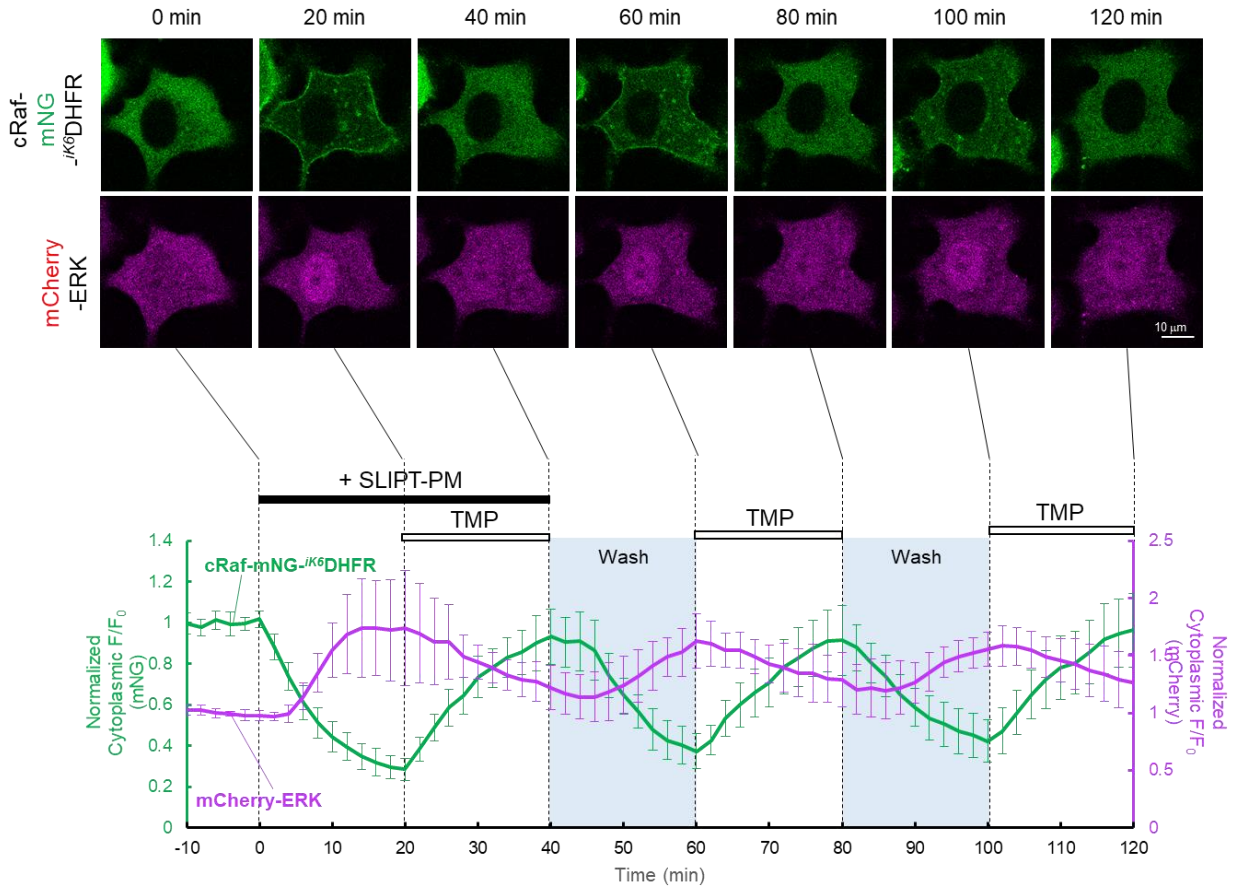
Reaction medium: serum-free DMEM

Experimental design:

cRaf, a downstream factor of Ras, can be activated by Ras on the PM and subsequently activate MEK-ERK pathway. Phosphorylated ERK is translocated into the nucleus and activates further downstream transcription factors. In this model experiment, ^{iK6}DHFR-fused cRaf protein (cRaf-mNG-^{iK6}DHFR) is translocated to the PM by SLIPT-PM and promotes activation of MEK-ERK pathway. Nuclear translocation of mCherry-ERK is monitored as a sensor of MEK-ERK pathway. After then, by addition of Free-TMP induced release of cRaf-mNG-^{iK6}DHFR from the PM to cytosol. Furthermore, wash out of Free-TMP reproduces PM-recruitment of cRaf-mNG-^{iK6}DHFR by remaining SLIPT-PM on the PM again and reactivates MEK-ERK pathway.



Before the addition of SLIPT-PM, both cRaf-mNG-^{iK6}DHFR and mCherry-ERK constantly localized in cytosol. By the addition of SLIPT-PM, firstly, cRaf-mNG-^{iK6}DHFR translocated to the PM ($t_{1/2}$~7 min) and subsequently, nuclear translocation of mCherry-ERK was observed. The addition of Free-TMP at 20 min clearly canceled the PM-tethering of cRaf-mNG-^{iK6}DHFR and nuclear localization of mCherry-ERK was decreased. The cell medium containing Free-TMP was continuously exchanged using a peristaltic pump from 40 min to 60 min recovered PM-translocation of cRaf-mNG-^{iK6}DHFR and nuclear localization of mCherry-ERK again without the addition of SLIPT-PM. In this experiment, reversible regulation of cRaf-mNG-^{iK6}DHFR and mCherry-ERK was successfully observed at least two cycles of Free-TMP addition and medium change. This result indicates SLIPT-PM is still tethering on the PM after Free-TMP addition and medium change.



*A time-lapse movie is available on our website

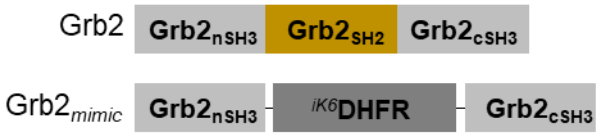
(7) Activation of Grb2/SOS1-Ras pathway

Target signaling pathway: Grb2/SOS1 → Ras → Raf → MEK → ERK

Constructs in the experiment

- ^{iK6}DHFR-fused protein: Grb2$_{mimic}$[*1]-iRFP713 (addgene ID: 178854)
  ^{iK6}DHFR-iRFP713 (addgene ID: 178855)

[*1] In this experiment, Grb2$_{mimic}$ indicates a chimeric protein which has ^{iK6}DHFR substituted to SH2 domain of Grb2.



- Reporter protein: FRET-type nucleus ERK activity sensor

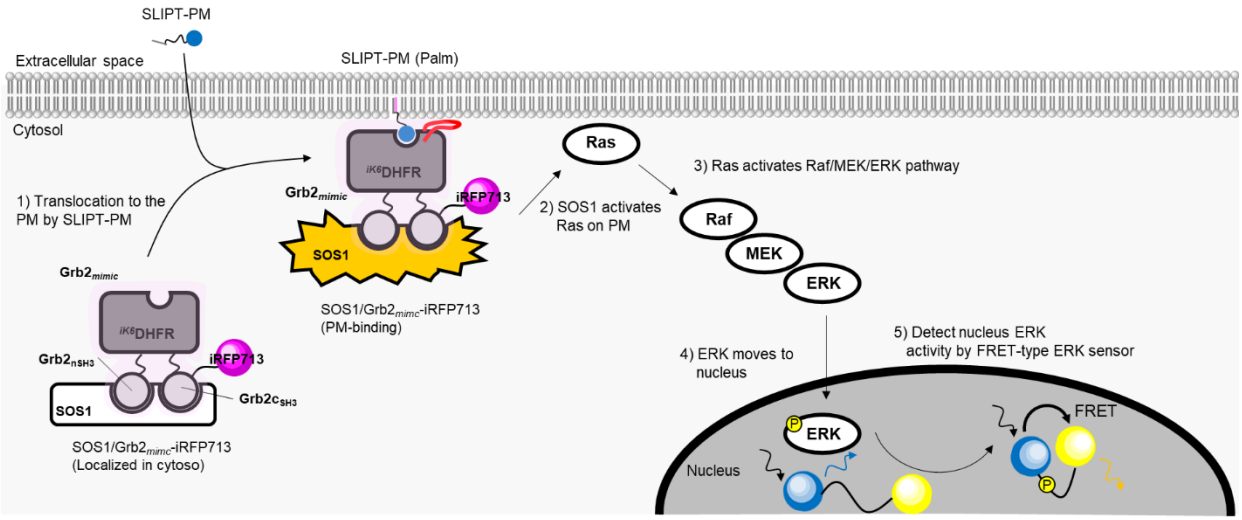
SLIPT-PM concentration: SLIPT-PM 5 μM

Cells: HeLa cells

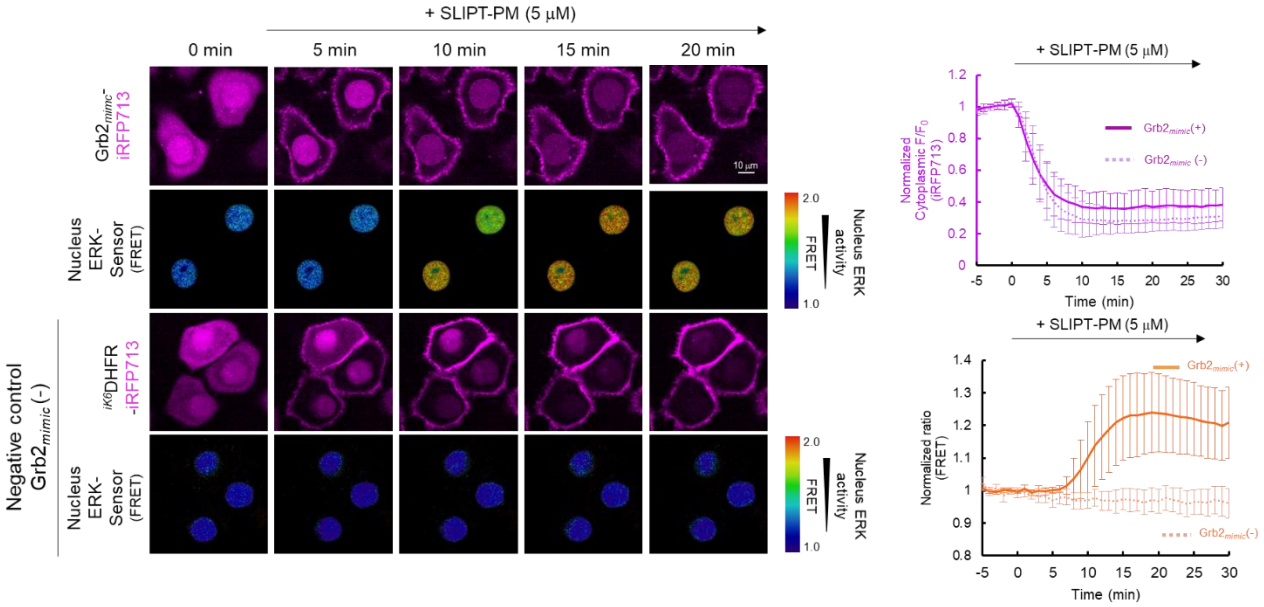
Reaction medium: serum-free DMEM

Experimental design:

Grb2 makes a complex with SOS1, a member of guanine nucleotide exchange factor for Ras (RasGEF), and the Grb2/SOS1 complex binds to intracellular domain of activated EGFR via SH2 domain of Grb2. Grb2/SOS1 complex on the PM activates Ras and its downstream pathway Raf-MEK-ERK. In this experimental model, a chimeric protein Grb2$_{mimic}$ which has ^{iK6}DHFR substituted to SH2 domain is used as ^{K6}DHFR-fused protein. Grb2$_{mimic}$ can binds to endogenous SOS1 via nSH3 and cSH3 and be recruited to the PM by SLIPT-PM. Grb2$_{mimic}$/SOS1 complex on the PM activated Ras-Raf-MEK-ERK pathway. Phosphorylated ERK activity in nucleus is monitored by the FRET-type ERK sensor.



Before the addition of SLIPT-PM, Grb2$_{mimic}$-iRFP713 and nucleus ERK sensor were constantly localized in cytosol and nucleus, respectively. By the addition of SLIPT-PM (5 μM), Grb2$_{mimic}$-iRFP713 rapidly translocated to the PM ($t_{1/2}$~5 min). After the PM recruitment of Grb2$_{mimic}$-iRFP713, FRET ratio was clearly changed. In contrast, ^{iK6}DHFR-iRFP713 as a negative control could quickly translocate to the PM, but no change of FRET ratio was observed. This result indicates the PM recruitment of Grb2$_{mimic}$-iRFP713 by SLIPT-PM can induce Ras-downstream signaling pathway.



*A time-lapse movie is available on our website

7. Original and reference papers

(1) Original paper of SLIPT system

Ishida *et al., J. Am. Chem. Soc.,* **135**, 12684-12689 (2013) Synthetic self-localizing ligands that control the spatial location of proteins in living cells.

(2) Original paper of SLIPT-PM (m^{D}cTMP)

Nakamura *et al., ACS Chem. Biol.*, **15**, 837-843 (2020) Designer palmitoylation motif-based self-localizing ligand for sustained control of protein localization in living cells and *Caenorhabditis elegans*

(3) Original paper of ^{iK6}DHFR/SLIPT-PM

Suzuki et al., *Cell Chem. Biol., in press* A chemogenetic platform for controlling plasma membrane signaling and synthetic signal oscillation.
[DOI: 10.1016/j.chembiol.2022.06.005]

(4) Related references

(4-1) Multiplex SLIPT system

Nakamura *et al, ACS Chem. Biol.*, **15**, 1004-1015 (2020) Engineering orthogonal, plasma membrane-specific SLIPT systems for multiplexed chemical control of signaling pathways in living single cells.

(4-2) The SLIPT ligand for endomembrane

Nakamura *et al., Biochemistry,* **59**, 205-211 (2020) Chemogenetic control of protein anchoring to endomembranes in living cells with lipid-tethered small molecules.

(4-3) The SLIPT ligand for Golgi apparatus

Suzuki *et al, Chem. Comm.*, **56**, 7961-7964 (2020) Golgi recruitment assay for visualizing small-molecule ligand-target engagement in cells.

8. Information

● DNA and amino acid sequences of ^{iK6}DHFR

<DNA sequence>

ATCAGTCTGATTGCGGCGTTAGCGGTAGATCGCGTTATCGGCATGGAAAACGCCATGCCGTGGAACCTGCCTGCCGATCTCGCCTGGTTTAA
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<Amino acid sequence>

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VPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERR

eDHFR internal K6-loop

Website : https://www.funakoshi.co.jp/exports_contents/95015

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