

A Method to Rapidly Induce Organelle-Specific Molecular Activities and Membrane Tethering

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Abstract

In this chapter we describe a technique for rapid protein targeting to individual intracellular organelles. This method enables a real-time imaging-based study of cellular behavior in response to controlled induction of signaling events in a specifically targeted cellular compartment. We provide rationales and a step-by-step protocol for probe design and imaging of protein targeting along with two different applications of this technique. One application involves organelle-specific activation of small GTPases, while the other application involves membrane tethering of two different organelles. In the former case, we activate Rac1 and Ras at distinct intracellular locations in order to study compartmentalization of their signaling pathways, and in the latter example, we induce membrane tethering of the endoplasmic reticulum and mitochondria in order to study organelle–organelle communication. The described technique allows to rapidly perturb molecular activities and organelle–organelle communications at precise locations with specified timing and represents a powerful strategy to dissect spatiotemporally complex biological processes.

Key words Cell signaling, Fluorescence imaging, Synthetic biology, Small GTPases, Membrane lipid signaling, Organelle–organelle interaction, Endoplasmic reticulum

1 Introduction

Many cellular signaling pathways are precisely localized and rapidly induced in response to environmental stimulation. Elucidation of the nature and functions of complex signaling networks requires development of cellular probes that operate in the same time scale as the signaling events themselves, act in well-defined cellular domains, and can be activated at defined time points [1]. A major strategy employed for this purpose involves a chemically induced heterodimerization to trigger rapid-onset and specific perturbations of various signaling molecules in living cells [2–8]. Since most signaling events compose networks operating at multiple intracellular locations [9], it is important to specifically induce signaling in distinct cellular organelles, such as plasma membrane, the Golgi complex, mitochondria, the endoplasmic reticulum (ER), or lysosomes.

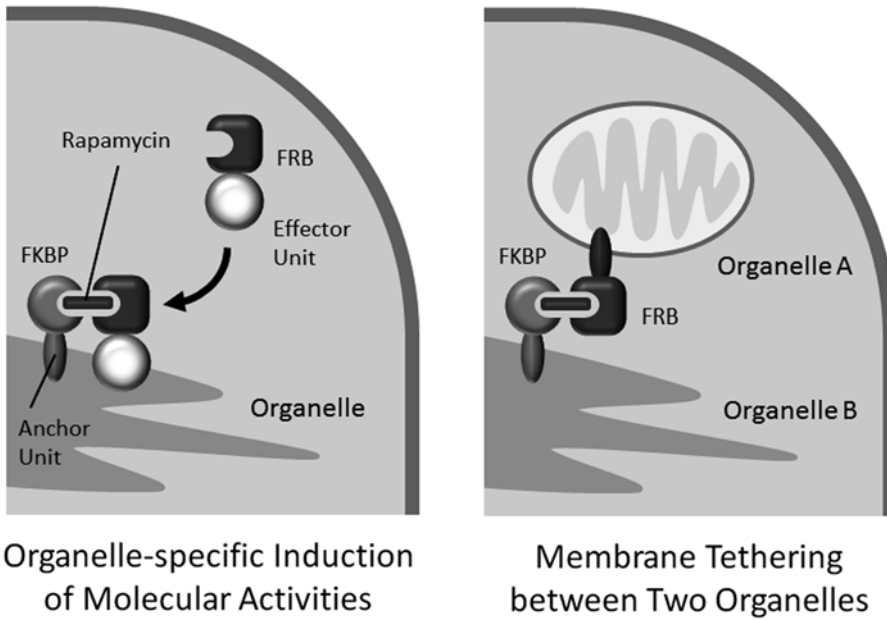


Fig. 1 Schematic illustration of the techniques described in this chapter

We achieved this goal in living cells via inducible dimerization of two engineered proteins triggered by addition of a chemical dimerizer and leading to the controlled organelle translocation of the proteins of interest. Specifically, we designed an anchoring unit that is attached to the cytoplasmic face of a specific organelle and an effector unit that is present in the cytosol and induces a signaling event upon translocation to the target membrane. Such translocation occurs via dimerization of the effector with the anchor, and it can induce site-specific signaling events on the organelle targeted by the anchoring unit (*see Fig. 1*, left). Furthermore, this technique can be used to dimerize two anchoring units (targeted to different organelles) in order to induce tethering of these organelles (*see Fig. 1*, right).

One commonly used example of a chemically induced dimerization system involves FK506-binding protein (FKBP) and FKBP-rapamycin-binding (FRB) domain, which can be dimerized by addition of rapamycin or its analogs such as indole rapamycin (*see Note 1*). Recent developments of this technique include photoactivatable dimerizers [10, 11], attempts to overcome a relative irreversibility of the standard FKBP-rapamycin-FRB system [12], and approaches to eliminate possible off-target effects of the method [13]. In this chapter, we describe the basic organelle-targeting technique and some of its cellular applications.

2 Materials

Prepare all solutions using analytical grade reagents under sterile conditions. Ensure that all relevant waste disposal regulations are followed when disposing waste materials.

2.1 Plasmid Design and Preparation

1. Vector DNAs: Plasmids for mammalian expression controlled by the cytomegalovirus (CMV) promoter. Single unit is encoded in a single plasmid (*see Note 2*).
2. cDNAs of proteins for the anchor unit and the effector unit.
3. Reagents for standard subcloning.

2.2 Cell Culture and Transfection (Preparation of Experiments)

1. Cultured cells (*see Note 3*): Maintain the cells at 37 °C in 5 % CO₂ atm at 90 % relative humidity in the appropriate cell culture medium.
2. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 60 U/mL penicillin, and 60 U/mL streptomycin.
3. Cell transfection medium: DMEM supplemented with 10 % FBS without antibiotics (*see Note 4*).
4. DMEM without FBS: DMEM without FBS is used in transfection mixture preparation. Plasmid transfection can also be performed using Opti-MEM reduced serum medium.
5. Trypsin-EDTA (*see Note 5*).
6. Phosphate-buffered saline (PBS), pH 7.4.
7. Plasmids: Plasmid DNA is prepared at 1 µg/mL concentration in sterile water.
8. Transfection reagents: In the described experiments, we used Lipofectamine 2000 (Invitrogen), but other transfection reagents can also be used.
9. Polylysine-coated cover slips: Sterilize round glass cover slips (0.1–0.2 mm thickness and 25 mm diameter) with absolute ethanol, air-dry them, and apply 30 µL of poly-D-lysine solution (0.1 mg/mL) per each cover slip. Incubate for 30 min at room temperature, and wash twice with sterile PBS (*see Note 6*).
10. Metal frame: Serves as a chamber to hold cover slips for cell imaging (Fig. 2).

2.3 Fluorescence Microscopy

1. Chemical dimerizer: Prepare a 1,000× stock solution of a desirable chemical dimerizer in dimethylsulfoxide (DMSO) (*see Notes 7 and 8*). Since 100 nM of rapamycin is sufficient to induce dimerization, its stock solution should be prepared at 100 µM concentration. Other chemical dimerizers should be prepared by using appropriate organic synthesis.

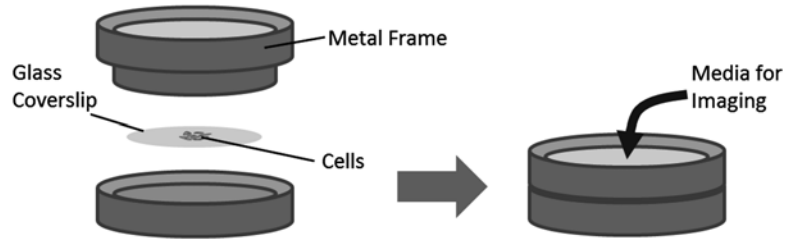


Fig. 2 Placement of cover slips in the metal frames

2. Physiological stimulant (such as growth factors or chemoattractants) (*see Note 9*): 1,000× Stock solution should be prepared following the manufacturer's instructions.
3. Fluorescent probes (such as calcium sensors and fluorescent lipids): 1,000× Stock solution should be prepared following the manufacturer's instructions (*see Note 10*).
4. Imaging media (*see Note 11*): PBS containing calcium and magnesium ions or phenol red-free DMEM.
5. Fluorescence microscope (*see Notes 12 and 13*): In our laboratory, live cell measurements are performed using a spinning-disc confocal microscope. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are excited with a helium-cadmium laser and an argon laser (CVI-Melles Griot, NM, USA), respectively. These two lasers are fiber-coupled to the spinning disc confocal unit (CSU10; Yokogawa, Japan) equipped with dual-CFP/YFP dichroic mirrors. Appropriate filter sets for CFP and YFP are used to enable capture of fluorescence images with a CCD camera (Orca ER, Hamamatsu Photonics, Japan). Images are taken using a 40× objective lens mounted on an inverted microscope (Axiovert 200, Carl Zeiss, Germany). The microscope is operated by the MetaMorph software package (Molecular Devices, CA, USA).
6. Data analysis software.

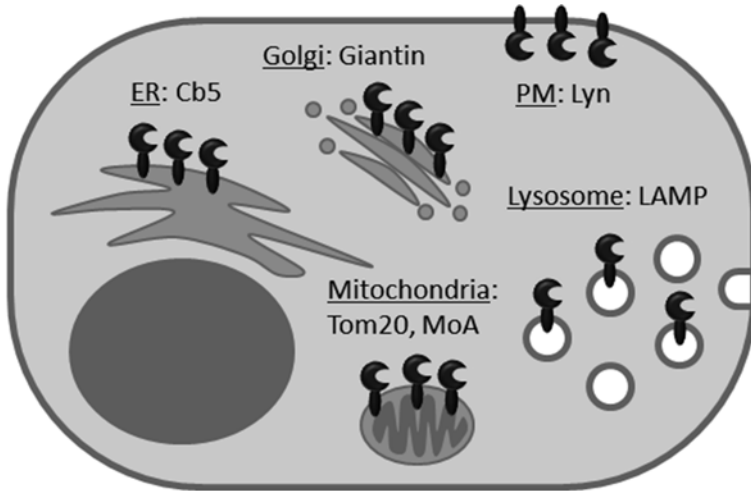
3 Methods

3.1 Plasmid Design and Preparation

The dimerization system is composed of an anchoring unit and an effector unit. The anchoring unit is designed to localize on the cytosolic face of a specific organelle, and the effector unit is designed to induce specific signaling events on the surface of the organelle targeted by the anchor. Plasmids for the anchoring and the effector units should be prepared by using standard subcloning protocols.

1. Construct design for the anchoring unit:

The design of the anchoring unit is based on proteins that are known to specifically localize in the target organelle. This unit also includes a fluorescent protein and one of the two dimerizing



Organelle	Name	Constructs
Plasma Membrane (PM)	Lyn	Lyn N-terminus sequence (GCIKSKGKDSA)
Golgi	Giantin	Giantin (3131-3259)
Endoplasmic reticulum (ER)	Cb5	Cytochrome b5 (100-134)
Lysosome	LAMP	Lysosomal-associated membrane protein 1 (1-417)
Mitochondria	Tom20 MoA	Tom20 (1-33) Monoamine Oxidase A (490-527)

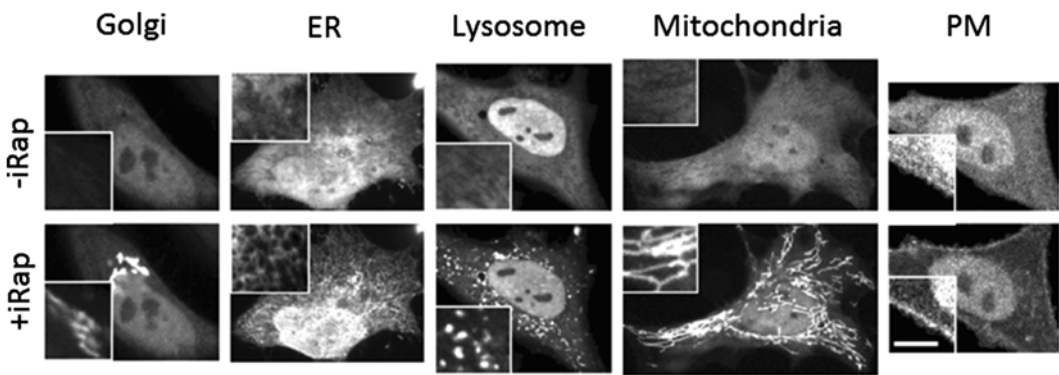


Fig. 3 Examples of the organelle-specific targeting constructs. Fluorescence images at the bottom show the translocation of cytosolic protein upon dimerization (+iRap) with the corresponding anchoring unit. Scale bar, 20 μm (scale bar in *insets*, 5 μm). The images are reproduced from [1] with permission

domains that are targeted to the cytosolic face of the organelle. The already designed organelle-targeting constructs are summarized in Fig. 3 [1]; however, it may be important to design novel organelle anchors. Here, we describe how to develop such a novel unit using the Golgi anchoring motif as an example.

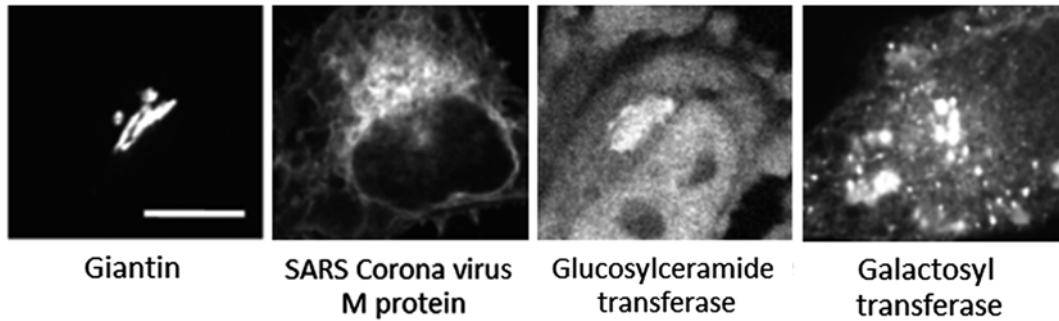


Fig. 4 Fluorescence images of candidate Golgi-targeting proteins. A giantin-based construct specifically localizes in the Golgi complex. By contrast, constructs based on SARS Corona virus M protein and glucosylceramide transferase demonstrate ER and cytosolic localization, respectively. Expression of a galactosyl transferase-based construct disrupts integrity of the Golgi complex. Scale bars, 20 μm . Reproduced from [1] with permission

Since it is impossible to predict the cellular localization of a particular protein based on its structural features, new anchors should be developed using a trial-and-error approach involving structural analysis of known organelle-resident proteins. To obtain motifs that specifically target the Golgi complex, we tried four different Golgi proteins and found that constructs derived from giantin work the best (Fig. 4). Giantin is a Golgi-specific structural protein [14] that is known to interact with multiple proteins to maintain the Golgi structure [15]. Since we intended to eliminate unwanted interactions between the anchoring unit and other proteins, we focused on the giantin sequence around its transmembrane domain (TMD). We found that a minimum sequence around the TMD is successfully targeted to the Golgi without disrupting morphology of this organelle. Therefore, this sequence was selected as the Golgi anchoring unit.

Next, we selected the dimerization domain and optimized the order of the dimerization domain and the fluorescent protein in the designed construct. These two parameters are known to affect the expression level of the anchor, the heterodimerization rate, and the structural features of the targeted organelle (*see Note 14*). Specifically, we swapped FKBP with FRB and placed FKBP or FRB at the N-terminal or the C-terminal ends of either the fluorescent protein or the anchoring motif. A construct with the following order, FKBP–(fluorescent protein)–(giantin TMD), appears to be the most suitable for our purposes.

2. Construct design for the effector unit:

The effector unit can be designed to perturb cellular signaling mediated by small GTPases [1, 2, 16, 17] or phosphoinositides [3, 16, 18]. Here we describe the design of the effector construct

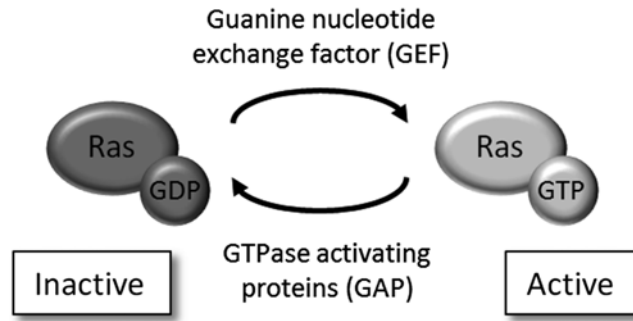


Fig. 5 The activity cycle of the Ras GTPase. Constitutively active Ras mutants have point mutations that block the GTP–GDP exchange. As a result, these mutants remain in the active state independently of cellular activities of their GEF and GAP

aimed to perturb GTPase signaling. GTPases are known to cycle between an active, GTP-bound and inactive, GDP-bound forms. The activation step is controlled by guanine nucleotide-exchanging factors (GEF), whereas the inactivation step is accelerated by GTPase-activating proteins (GAP) (Fig. 5). Based on this activation–inactivation cycle two different strategies for modulating GTPase signaling have been designed. One strategy is to create a constitutively active GTPase that is not regulated by GEF and GAP, and the other strategy is to use a specific GEF for the targeted GTPase. The first approach was used to recruit constitutively active forms of Rac1, Cdc42, and RhoA to the cellular membranes [2]. The second, more natural approach to activate endogenous GTPases was used to target Rac1 [2, 17], Ras [1], and Arf6 [16]. The design of the effector unit involves removing domains that determine effector compartmentalization to ensure its localization in the cytosol prior to dimerization. Afterwards, dimerization domains and fluorescent proteins are attached in various orders and pairings, and the activity of the constructs is tested.

3. Plasmid preparation:

Prepare the plasmids according to standard subcloning protocols using the design strategy described above. Validate all plasmids by sequencing.

3.2 Cell Culture and Transfection (Preparation of Experiments)

1. To study organelle-specific cellular events and membrane tethering, select plasmid pairs containing appropriate anchoring and effector units. To study the morphology and functions of the targeted organelle, prepare required protein biosensors (*see Note 15*), antibodies for immunolabeling (*see Note 16*), and small molecular fluorescent probes, such as calcium sensors and fluorescent lipids (*see Note 17*).

2. Pre-warm culture media, DMEM with FBS and without antibiotics, trypsin, and DMEM without FBS at 37 °C.
3. Prepare the transfection mixture in a 1.5 mL sterile microcentrifuge tube. Its composition depends on the transfection reagents used. For Lipofectamine 2000 transfection, dilute up to 2 µg (normally 0.3–1.2 µg) of plasmid DNA and 4.5 µL Lipofectamine in 100 µL DMEM without FBS. If multiple constructs are transfected, their ratio should be optimized by testing expression of each construct.
4. Incubate the transfection mixture for 20 min at room temperature.
5. In the meantime, prepare glass cover slips coated with poly-D-lysine.
6. Trypsinize cells, transfer them into 15 mL tube using 10 mL of the culture medium, and spin them down (1,000 × *g* for 3 min).
7. Resuspend the cells into 1 mL culture media for transfection (per condition).
8. Add 1 mL of resuspended cells to 100 µL of the transfection mixture, and mix them well by gentle pipetting.
9. Add 50 µL of the cell suspension onto each cover slip.
10. Leave the cover slips in the incubator for 1 h (*see Note 18*).
11. Aspirate the media, and add 1 mL of fresh culture media for transfection.
12. Acquire images 24 or 48 h post-transfection.

3.3 Fluorescence Microscopy

1. Wash cover slips twice with PBS, and place them into metal frames filled with the imaging medium. The medium should not contain chemical dimerizer that should be added only during the fluorescence imaging.
2. Use confocal microscopy for image acquisition. Usually a single Z-point (preferably around the center of the cells), where the translocation and following signaling events can be clearly visualized, is selected for the imaging. For short imaging times (<30 min), neither incubation at 37 °C nor CO₂ are necessary, but these conditions are desirable for longer imaging in order to preserve cell viability.
3. Under the microscope, select the cell(s) for monitoring in the live imaging mode. These cells should contain all the constructs required for the perturbation and monitoring, which should be confirmed by checking appropriate fluorescence signals. Cells that are unusually dim or bright, or that show aberrant localization of the construct, should not be selected (*see Note 19*).
4. Start the image acquisition in a time-lapse mode. The time frame should be determined depending on the event to be monitored.

Usually, translocation is completed within 10 s after addition of chemical dimerizer, so images are generally acquired every 10 or 15 s.

5. After time-lapse imaging has been started, add the chemical dimerizer. It is important to minimize the focus drift during addition of chemical dimerizer. We achieve this by removing half of the medium (500 μ L in the case of imaging in 1 mL volume) from the dish by mechanical pipette, mixing it with the chemical dimerizer, and then gently returning it back into the dish (*see Note 20*). Then, the dimerizer rapidly reaches cells by diffusion, so the translocation should begin immediately. The microscope operating software should be used to monitor the timing of stimuli for subsequent data analysis.
6. Acquire images until the event of interest is completed.

3.4 Data Analysis

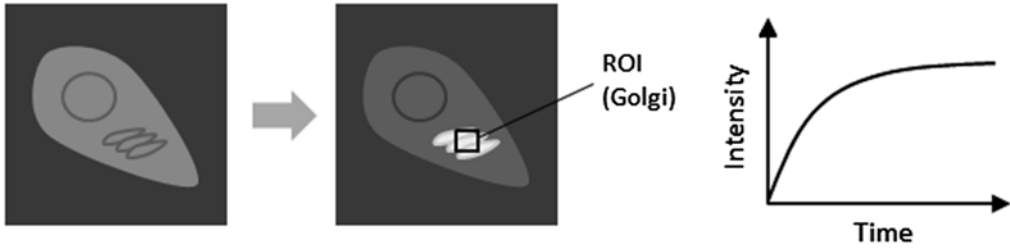
1. Use the image acquisition software or other image analysis software for the data analysis. Evaluate translocation of constructs and biosensors by taking regions of interest (ROIs) and calculating the fluorescence signal intensity in these regions (Fig. 6, top and middle).
2. Alternatively, quantify the probe translocation by measuring the decrease of its fluorescence intensity in the cytosol (*see Fig. 6*, bottom). The latter approach is useful to examine protein translocation to irregular compartments or small or thin organelles, where quantification may be difficult due to cellular movement. For example, translocation of Ras-binding protein to the Golgi complex can be examined by monitoring the increase in fluorescence intensity of the Golgi. On the other hand, translocation to the plasma membrane is better analyzed by monitoring the decrease in the cytosolic fluorescence, because the plasma membrane is thin, and its fluorescence measurements suffer from high noise level due to ROI drift.

3.5 Examples of Applications

1. Compartmentalization of Ras signaling:
Ras localizes on various membranes of living cells, and its activation has been observed at the plasma membrane and/or the Golgi complex in T cells, depending on the type of stimulus [9]. To distinguish between different membrane pools of Ras, we designed the organelle-specific anchoring units together with the effector unit consisting of a guanine nucleotide exchange factor for Ras (RasGEF). This allowed us to investigate the output of compartmentalized Ras signaling.

We detected formation of membrane ruffles a few minutes after targeting of active Ras to the plasma membrane (Fig. 7). Oppositely, targeting of active Ras to the Golgi complex did not cause changes in plasma membrane dynamics. This data suggests that local activation of Ras at the plasma membrane,

Golgi Translocation



Plasma Membrane (PM) Translocation

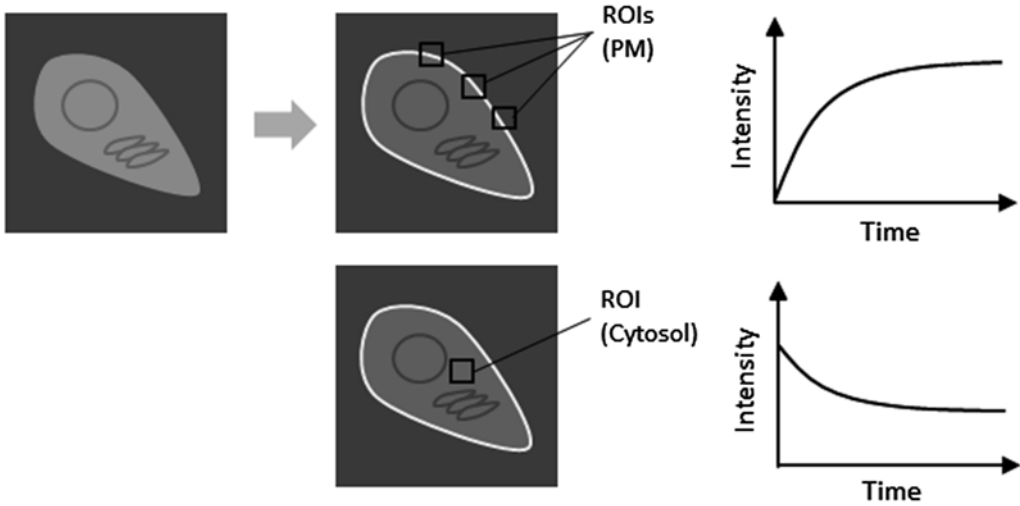


Fig. 6 Quantification of the probe translocation to the cellular membranes. Due to limited expression of the constructs containing fluorescent protein, their translocation from the cytosol to the target compartment results in a decrease of fluorescence in cytosol. In case of the thin plasma membrane, such translocation can be quantitated by using the average of multiple ROIs to reduce the error or by monitoring the decrease in fluorescence intensity in the cytosol

but not at the cell interior (Golgi), regulates remodeling of the cortical actin filaments. The same system has been used to study downstream signaling, such as ERK phosphorylation.

3.5.1 Membrane Translocation During Organelle–Organelle Tethering

To rapidly link mitochondria and ER (thus mimicking mitochondria-associated membranes, MAMs [19]) in living cells, we expressed the anchoring units for both ER and mitochondria and then induced their heterodimerization to connect the two membranes. As shown in Fig. 8, addition of the dimerizer to cells co-expressing two anchoring units induced a rapid transformation of the typical meshwork-like structure of ER to a tubular shape that is more typical of mitochondria. The resulting synthetic MAMs showed an accumulation of fluorescent dye-labeled phosphatidylserine [20] at the interface between the two organelles, indicating the formation of the unique membrane structures.

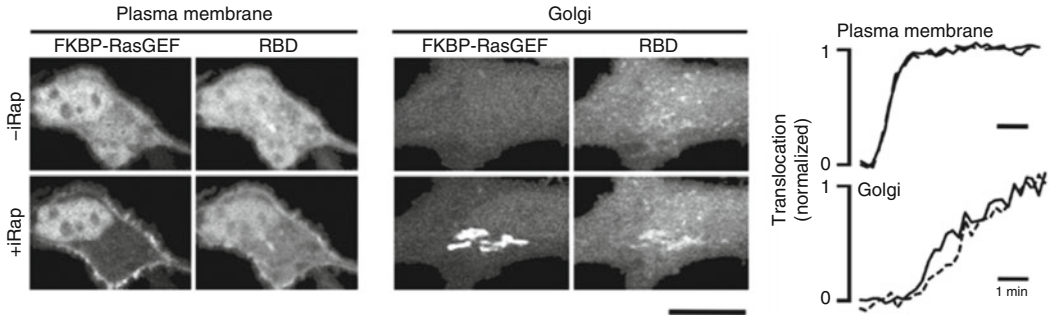


Fig. 7 Observation of the compartmentalized Ras signaling. Recruitment of FKBP-RasGEF to the plasma membrane or the Golgi complex upon addition of chemical dimerizer (+iRap) resulted in Ras activation only on the corresponding organelle, which can be monitored by translocation of the fluorescent protein modified with Ras-binding domain (RBD). Membrane ruffling is observed only after Ras activation to the plasma membrane. Scale bar, 20 μ m. Reproduced from [1] with permission

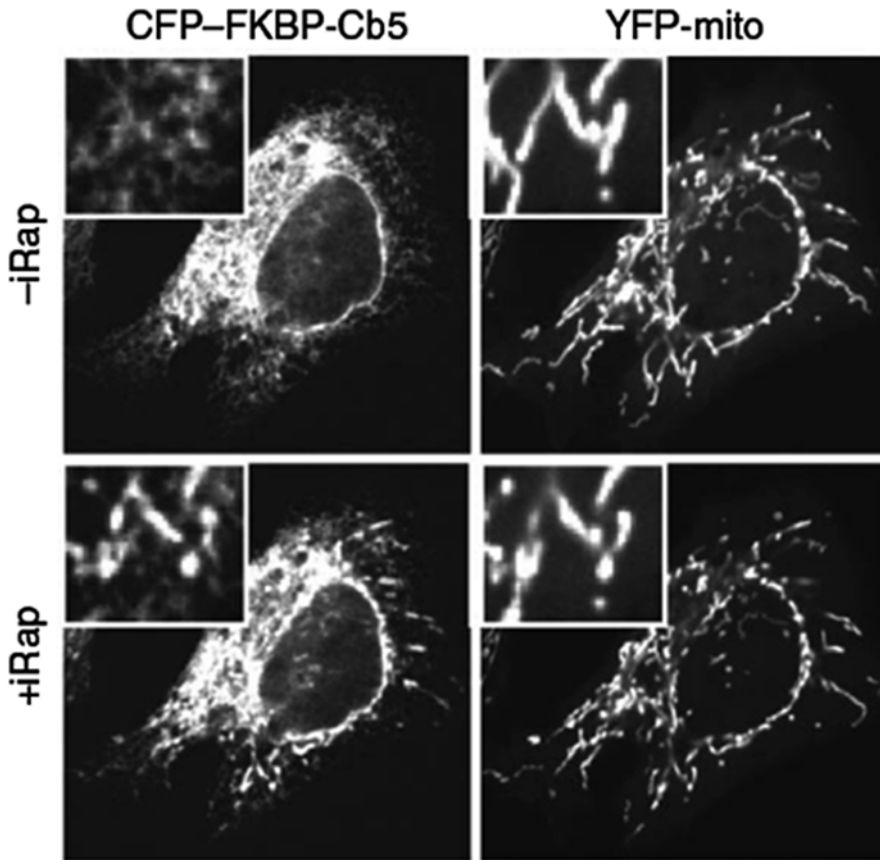


Fig. 8 Tethering of mitochondrial and ER membranes. After addition of the chemical dimerizer (+iRap), ER structure (imaged with CFP-FKBP-Cb5) shows a dramatic transformation reflecting formation of a novel interface between the ER and mitochondria (imaged with YFP-mito). Reproduced from [1] with permission

4 Notes

1. Dimerization systems that are orthogonal to the rapamycin-based system have been reported, such as gibberellin-based heterodimerization. When using the rapamycin-induced system, one should be aware that rapamycin can inhibit mTOR activity, thereby perturbing downstream signaling events [21]. However, these downstream effects of mTOR activation require hours to develop; therefore, they will unlikely interfere with cellular events that occur on a time scale of seconds to minutes. There are alternative chemical dimerizers, such as iRap, which does not affect the mTOR signaling [4].
2. In order to visually confirm the transfection and quantify the translocation, each construct should contain a fluorescent protein, such as CFP, YFP, or mCherry, in addition to the dimerization domain. Since both the anchoring and the effector units are required for perturbation, cells are transfected with a mixture of two constructs that contain the respective dimerization domains and differently colored fluorescent proteins. If the signaling events need to be monitored with fluorescent protein-based biosensors, these sensors are also expressed in the cells. In this case, the anchoring unit is designed without a fluorescent protein to allow tagging this fluorescence protein with the biosensor.
3. The system can be applied to any cultured cells as long as the constructs can be expressed. For example, the tethering systems appeared to be useful in model neutrophils to study the role of phosphoinositide signaling in chemotaxis [15]. In this chapter, we focus on commonly used mammalian cell lines, such as HeLa, NIH3T3, HEK 293, and COS7 cells, for all of which the system has been successfully used.
4. The use of the lipofection reagents with antibiotics can cause cytotoxicity due to the internalization of antibiotics. Therefore, transfection should be performed in the antibiotic-free medium.
5. The optimal concentration of trypsin is determined by the tightness of cellular adhesion. Most cell types can be detached with 0.05 % trypsin, but highly adherent cells such as MDCK require 0.25 % trypsin.
6. Alternatively, commercially available glass-bottomed dishes or chambered cover slips pre-coated with poly-D-lysine can be used.
7. The final concentration of DMSO in the cell culture medium will be 0.1 %. This level of DMSO is well tolerated by the majority of cell lines. For the DMSO-sensitive cell lines (such as neural cells) the final vehicle concentration can be reduced. Adding the same concentration of DMSO to all appropriate controls is essential.

8. The stock solutions should be divided into single-use aliquots that are stored at $-20\text{ }^{\circ}\text{C}$ to avoid repeated freeze-and-thaw cycles. During the experiment, the aliquots should be kept on ice before use.
9. Since membrane targeting experiments are usually performed to better understand different steps of the signaling pathways induced by external stimuli, such as growth factors, it is desirable to compare the effects of chemical dimerizers with the effects of physiological stimuli. Such stimuli may also be used as a positive control for cellular responses and biosensors.
10. Many lipophilic sensors are difficult to solubilize and being introduced into the cell. To prepare stock solutions of such sensors, the manufacturer's instructions should be strictly followed. In general, lipid solutions in organic solvents are dried and then reconstituted in buffers containing defatted BSA with rigorous vortexing, and the resulting BSA-lipid complexes are diluted to the appropriate concentration before adding to the cells.
11. Since growth factors and nutrients in FBS can cause nonspecific signaling in the transfected cells, the imaging experiments should be performed in the FBS-free medium.
12. A confocal microscope is more suitable than an epifluorescence microscope to monitor and quantify protein translocation. Any confocal microscope can be used as long as it can detect differently colored fluorescent proteins independently, and the image acquisition speed is fast enough relatively to the targeted cellular response.
13. A multi-positioning scanner on the microscope makes it possible to monitor signaling events in multiple cells on the dish simultaneously. Monitoring a larger number of cells is desirable in order to increase the reliability of results and identify both false-positive and false-negative events.
14. These effects can be evaluated by measuring fluorescence intensities of the constructs, the responses to chemical dimerizers, the fluorescence images of co-expressed organelle markers, and viability of the transfected cells. Different responses may be due to differences in protein stability and/or the steric environment of the FKBP-FRB interaction.
15. Fluorescent biosensors can be used to visualize signaling events induced by the organelle-specific manipulations described above. Several biosensors are available to monitor live cell activity of small GTPases. For example, Ras activation can be examined by a fluorescent protein attached to the Ras-binding domain (RBD) that specifically interacts with active Ras [22]. Additionally, several FRET-based biosensors for small GTPases have been developed [23].

16. If the appropriate biosensor is not available, the downstream signaling events can be studied by immunofluorescence labeling with phospho-specific antibodies. Since transfection efficiency can vary from cell to cell, and some cells might show either high or low level of construct expression, the signaling events should be analyzed in the cells with moderate level of expressed reporters.
17. A key question in studying organelle-to-organelle communications is how the interaction causes redistribution of the molecules of interest. Phospholipids are important messengers, and their translocation during organelle–organelle interactions can be studied by using the membrane-tethering system and commercially available fluorescently conjugated lipids [20]. For example, we used fatty acid-labeled phosphatidylserine (Avanti Polar Lipids, Inc., AL, USA) to monitor phospholipid translocation at junctional sites of ER and mitochondria upon organelle tethering.
18. The incubation time should be exactly 1 h.
19. During the search for cells to image, care should be taken to limit light exposure, and avoid photobleaching of the constructs or photodamages of the cells.
20. This also helps to preserve the temperature before and after addition of the chemical dimerizers to avoid the focus drift arising from temperature changes.

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