

Award Accounts

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Genetically Encoded Fluorescent Indicators to Visualize Molecular Processes in Living Cells

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Fluorescence imaging could be the most powerful technique available for observing spatial and temporal dynamics of molecular processes in living cells, if fluorescent indicators for the relevant molecular processes become available. Therefore, we have been developing fluorescent indicators for a variety of cellular signaling processes, including second messengers and protein phosphorylation reactions. Using the indicators, we have visualized spatial and temporal dynamics of these molecular events in single living cells. The present fluorescent indicators are becoming an indispensable tool for understanding the complex mechanism of the signal transduction in living cells and for screening pharmaceuticals that inhibit or promote molecular processes in the cell.

1. Introduction

Breakthroughs in understanding signal transduction in biological systems, such as the central nervous and endocrine systems, depend on decoding the spatial and temporal dynamics of intra- and extracellular signaling molecules. The most generally applicable and popular techniques with high spatial and temporal resolutions are optical ones. In 1985, a synthetic fluorescent Ca^{2+} indicator, named Fura-2, has been developed by Tsien et al.[1] Fura-2 has been used to uncover the complex dynamics of Ca^{2+} in single living cells, such as Ca^{2+} waves and Ca^{2+} oscillations.[2] In addition to Fura-2 for Ca^{2+}, synthetic fluorescent indicators have been developed for several ions and small molecules, including Na^+, K^+, Zn^{2+}, and nitric oxide.[3,4] However, I felt a limitation of the organic synthesis-based approach to the development of fluorescent indicators when I was a Ph.D. course student. Biomolecules that regulate physiological and/or pathophysiological processes in the cell, such as proteins, lipids, and sugar chains, have structures that are too complicated to develop indicators using organic synthesis. Thus, we have been developing fluorescent indicators using protein domains that specifically recognize biomolecules having complicated structures. We also have uncovered spatial and temporal dynamics of molecular processes in living cells using our indicators. In this Award Account, I present several examples of our genetically encoded fluorescent indicators to visualize key molecular processes in living cells.

2. Fluorescent Indicators for Protein Phosphorylation

Protein phosphorylation plays one of the most pivotal roles in signaling pathways within cells.[5] Hundreds of different protein kinases that catalyze protein phosphorylation are organized into complex networks of signaling pathways in the cell to control diverse cellular functions from survival to apoptosis. Protein phosphorylation is also involved in a variety of pathophysiologic states, including cancer, inflammatory disorders, and cardiac diseases. However, existing methods, including electrophoresis, immunocytochemistry, and in vitro kinase assay, do not give any spatial and temporal information about where and when protein kinases are activated to phosphorylate substrate proteins in living cells. To overcome the limitations of the existing methods, we have developed genetically encoded fluorescent indicators that allow to visualize the spatial and temporal dynamics of protein phosphorylation in living cells (Fig. 1a).[6]

We fused a substrate sequence of a protein kinase with a phosphorylation recognition domain via a flexible linker sequence to afford a fluorescent indicator for protein phosphorylation (Fig. 1a). The tandem fusion unit was further sandwiched with cyan fluorescent protein (CFP)[7] and yellow fluorescent protein (YFP),[7] which serve as the donor and acceptor fluorophores for fluorescence resonance energy transfer (FRET), respectively. As a result of phosphorylation of the substrate sequence, the adjacent phosphorylation recognition domain bound to the phosphorylated substrate sequence in the fluorescent indicator. Then, FRET occurs from the donor CFP to the acceptor YFP, which elicits a phosphorylation-dependent change in the emission ratio of the donor and acceptor fluorophores (Figs. 1a and 1b). Upon activation of phosphatases, the phosphorylated substrate domain of the fluorescent indicator is dephosphorylated, and the FRET signal decreases. We named this fluorescent indicator "Phocus" (a fluorescent indicator for

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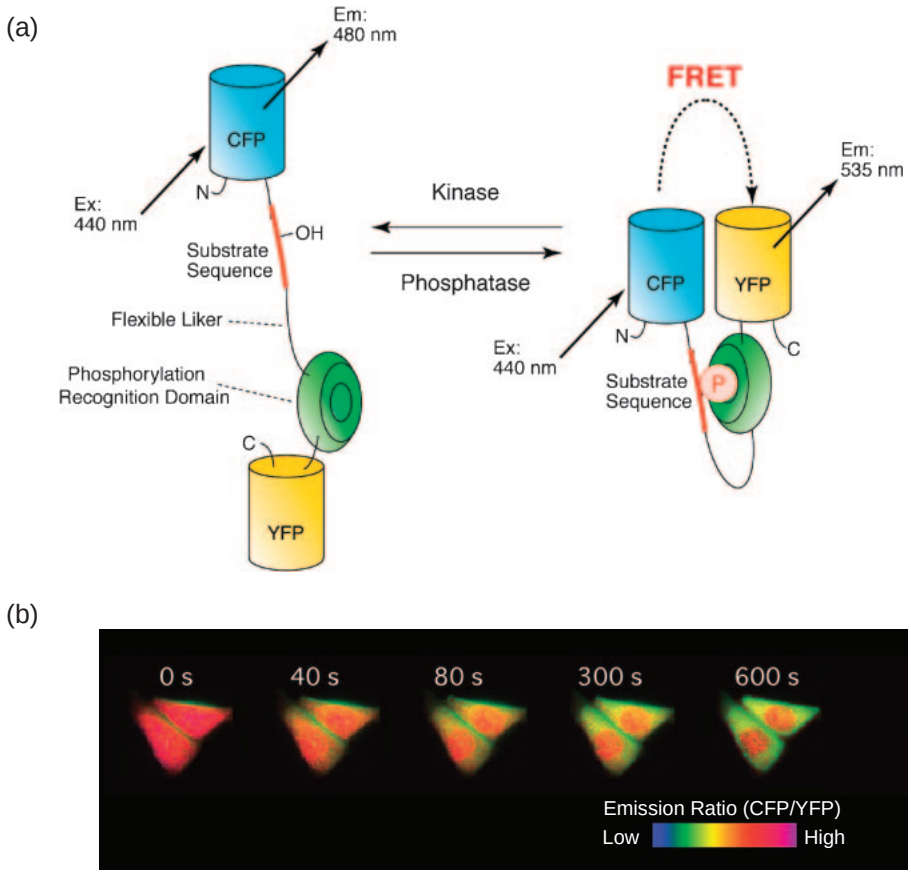

Fig. 1. Fluorescent indicator for protein phosphorylation. (**a**) Principle of the present fluorescent indicator for protein phosphorylation, named "Phocus." (**b**) Fluorescence imaging of protein phosphorylation in living cells by insulin receptor tyrosine kinase. We developed and used a Phocus variant having a substrate sequence that is phosphorylated by insulin receptor. We used an SH2 domain from phosphatidylinositol 3-kinase for the phosphorylation recognition domain of the Phocus variant for insulin receptor. Pseudocolor images of the CFP/YFP emission ratio are shown before and 40, 80, 300, and 600 s after the addition of 100 nM insulin at 25 °C. The fluorescent images were taken by using an epifluorescence microscope. The cell was excited at 440 ± 10 nm. Fluorescence images were obtained through 480 ± 15 and 535 ± 12.5 nm filters with a 40× oil immersion objective.

protein phosphorylation that can be custom-made).[6] The substrate sequence is the key determinant of the specificity of Phocus. We developed several variants of Phocus so far by using substrate sequences respectively selective to different protein kinases, such as tyrosine kinases (insulin receptor,[6] epidermal growth factor receptor, and Src[8]) and serine/threonine kinases (Akt,[9] ERK,[10] and JNK). The present indicator Phocus is thus flexible to a wide variety of different protein kinases, including tyrosine kinases and serine/threonine kinases.[11]

2.1 ERK. Extracellular signal-regulated kinase (ERK) is a serine/threonine protein kinase that regulates a wide variety of cell functions, such as cell growth and differentiation.[12] We developed a Phocus variant for ERK, named Erkus, using an amino acid sequence of KRELVEPLT^{669}PSIEAPNQALLR for the substrate sequence. This substrate sequence contains threonine residue 669 that is preferentially phosphorylated by ERK. We used a forkhead-associated 2 (FHA2) domain[13] derived from yeast Rad53p for the phosphorylation recognition domain of Erkus. We then connected a nuclear localization sequence (NLS) and a nuclear export sequence (NES) to Erkus to examine nuclear and cytosolic dynamics of protein phosphorylation by ERK, respectively. When expressed in MCF-7 cells, Erkus-nuc was localized in the nucleus of the cells (Fig. 2a, right panel). Erkus-cyto was excluded from the nucleus and localized in the cytosol of the MCF-7 cells, as expected (Fig. 2a, left panel). Thus, Erkus-nuc visualizes the ERK activity in the nucleus, whereas Erkus-cyto visualizes the cytosolic activity of ERK in the cells.

We found that the activation duration of ERK was considerably different between the cytosol and nucleus in living cells (Fig. 2b). The cytosolic ERK activity was shown to be transient upon stimulation with epidermal growth factor (EGF) due to the inactivation of the activated ERK by phosphatases in the cytosol. In contrast, the nuclear activity of ERK was longer than 60 min upon EGF stimulation. The present measurement of the subcellular ERK activity in the cytosol and nucleus shows that ERK substrates located in the cytosol are transiently phosphorylated, whereas those in the nucleus remain phosphorylated longer than 60 min. Nuclear ERK substrates include several transcriptional factors that play a key role in the gene expression.[12] The sustained activation of ERK in the nucleus, which was shown using Erkus-nuc, accompanies sustained gene expression to regulate the cell functions, including cell proliferation and differentiation.

2.2 Akt. Akt is a serine/threonine kinase, which regulates a variety of cellular responses, such as cell proliferation, cell

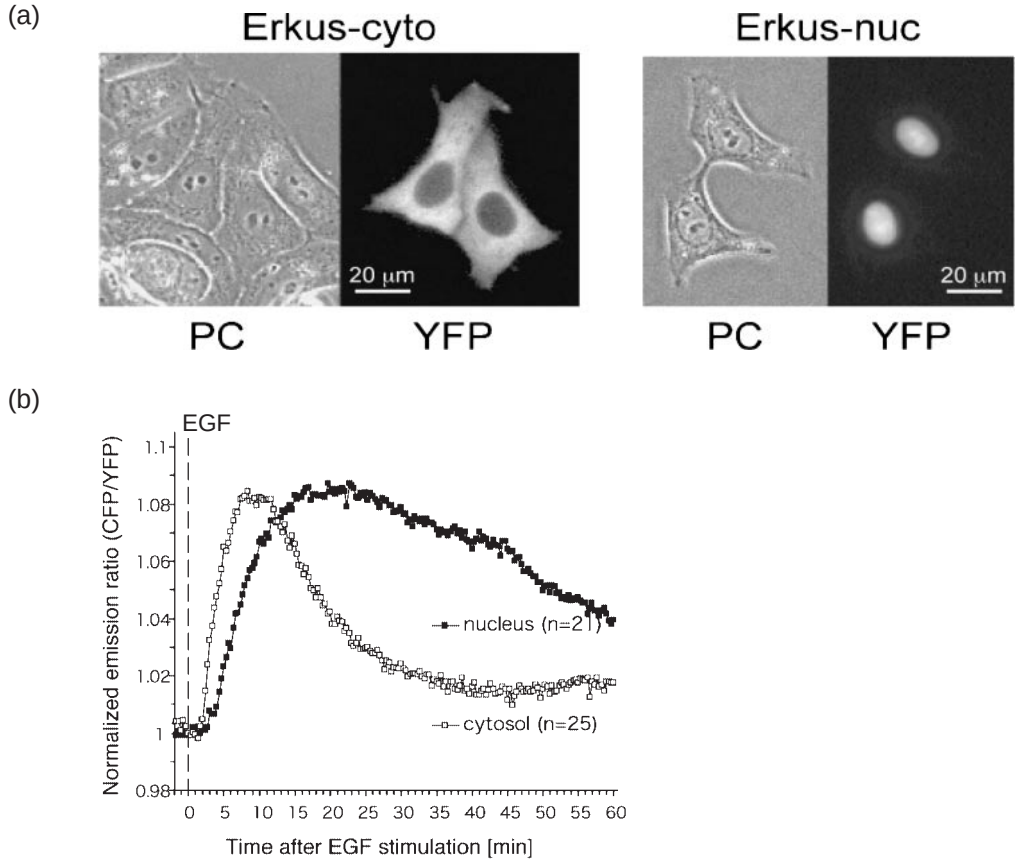

Fig. 2. Imaging protein phosphorylation by ERK using Erkus. (**a**) Subcellular distribution of Erkus-cyto (left panel) and Erkus-nuc (right panel) in MCF-7 cell. (**b**) Time course of the Erkus-nuc or Erkus-cyto response in MCF-7 cells upon stimulation with 100 ng mL^{-1} EGF. We found that Erkus showed an increase in the CFP/YFP emission ratio upon phosphorylation by ERK, in contrast to the Phocus variant for insulin receptor that showed a decrease in the emission ratio upon phosphorylation. This appears to be due to the different conformations of the FHA2 domain of Erkus and the SH2 domain of the Phocus variant for insulin receptor.

survival and angiogenesis.[14] To provide information about the spatial and temporal dynamics of the Akt activity in living cells, we developed genetically encoded fluorescent indicator for Akt, named Aktus.[9] Like the Erkus variants, we fused subcellular localization sequences with Aktus, because endogenous substrate proteins of Akt are often localized in subcellular regions of the cell. For example, eNOS,[15] which mediates a vasodilatory effect via production of nitric oxide, is predominantly localized in the Golgi apparatus membrane, whereas Bad,[16] which is related to apoptosis promotion, is present in mitochondrial outer membranes. By fusing the Aktus with the respective subcellular localization domains within the eNOS and Bad, Golgi-Aktus and Mit-Aktus, which are localized in the Golgi apparatus and mitochondrial outer membranes, respectively, were developed. In addition, these two Aktus variants were compared with a cytosolic diffusible indicator, Aktus, having no localization sequence. We showed that, in vascular endothelial cells, the Golgi-localized indicator, Golgi-Aktus, was phosphorylated upon stimulation with a peptide hormone, insulin, and with a steroid hormone, 17β-estradiol, whereas the mitochondria-localized Mit-Aktus was phosphorylated with 17β-estradiol but not with insulin (Table 1). On the other hand, the cytosolic indicator, Aktus, was not efficiently phosphorylated either upon stimulation with either insulin or 17β-estradiol (Table 1). These results indicate that activated Akt is localized in various subcellular compartments, including the Golgi apparatus and/or mitochondria, rather than diffusing throughout the cytosol, thereby efficiently phosphorylating its substrate proteins. Our observations with the mitochondria-localized indicator suggest that localization of activated Akt to mitochondrial outer membrane is triggered by 17β-estradiol but not by insulin. The present indicators and their applications thus should contribute to the studies of a whole range of dynamics of activated Akt in living cells.

Table 1. Differential Subcellular Localization of Activated Akt between Cellular Stimuli

	Cytosol[a)]	Golgi membrane[b)]	Mitochondrial outer membrane[c)]
Insulin	−	+	−
17β-Estradiol	−	+	+

a) Measured with Aktus. b) Measured with Golgi-Aktus. c) Measured with Mit-Aktus.

3. Fluorescent Indicators for Lipid Second Messengers

3.1 Phosphatidylinositol 3,4,5-Trisphosphate. Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is a lipid second messenger, which regulates diverse cellular functions, including cell proliferation and apoptosis, and is also related to diabetes, can-

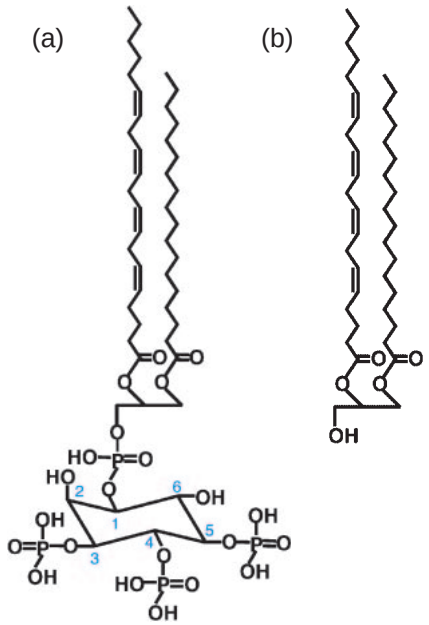

Fig. 3. Lipid second messengers. (**a**) Chemical structure of phosphatidylinositol 3,4,5-trisphosphate. (**b**) Chemical structure of diacylglycerol.

cer, etc. (Fig. 3a).[17] PIP_3 is generated at the cellular membrane by phosphoinositide 3-kinases (PI3Ks), and recruits and activates its binding proteins at the cellular membranes, including a protein kinase Akt, 3-phosphoinositide-dependent protein kinase-1 (PDK1), Bruton's tyrosine kinase (Btk) and general receptor for phosphoinositide-1 (GRP1). However, little is known about exactly when, where, and how PIP_3 is produced. This appeared to be due to the lack of appropriate methods to quantitatively analyze the spatial and temporal dynamics of PIP_3 in single living cells. Therefore, we developed genetically encoded fluorescent indicators for PIP_3, based on FRET (Fig. 4).[18] These novel PIP_3 indicators were composed of two different-colored mutants of GFP and a PIP_3-binding domain, and the PIP_3 level was observed by dual-emission ratio imaging, thereby allowing for stable observation of PIP_3 in single living cells. We used a pleckstrin homology domain (PHD) of GRP1,[19] which selectively binds with PIP_3 for the construction of the fluorescent indicators. The PHD was sandwiched between CFP and YFP via rigid α-helical linkers, consisting of repeated EAAAR sequences. Within one of the rigid linkers, a single diglycine motif was introduced as a hinge. This chimeric reporter protein was tethered to the membrane by fusing it to a membrane localization sequence (MLS) via the rigid α-helical linker. When PIP_3 was produced at the membrane upon PI3K activation, the PHD of the reporter protein bound it. Then, a significant conformational change of the reporter protein should take place due to the flexible diglycine motif introduced in the rigid α-helical linker. This flip–flop-type conformational change of the reporter protein is expected to result in intramolecular FRET from CFP to YFP, which makes it possible to detect the dynamics of PIP_3 at the cellular membrane (Fig. 4). We named this indicator "Fllip" (a fluorescent indicator for a lipid second messenger that can be tailor-made).[18]

Fllip can be located to particular membrane surfaces of interest by connecting appropriate MLSs, such as lipidation sequences and transmembrane sequences. The CAAX box motif[20] of N-Ras as the MLS yielded a reporter for PIP_3 at the plasma membrane (Fllip-pm). Using the mutated CAAX box sequence[20] of N-Ras, in which the cysteine 181 was replaced with a serine, Fllip was localized at the endomembranes to monitor the PIP_3 level there (Fllip-em). Using Fllip variants that exhibit each specific subcellular distribution, we elucidated the spatio-temporal regulations of PIP_3 production in single living cells. We found that in response to ligand stimuli, the amount of PIP_3 increased to a large extent at the endomembranes rather than at the plasma membrane (Figs. 5a–5c). In addition, we showed that the PIP_3 increase at the endomembranes was due to its in situ production at the endomembranes triggered by endocytosed receptor tyrosine kinases. The demonstration of PIP_3 production through receptor endocytosis addresses a long-lasting question about how its downstream signaling pathways including Akt are activated in intracellular compartments remote from the plasma membrane, such as the Golgi and mitochondria.

From a methodological viewpoint, the present method has general applicability for a wide variety of lipid second

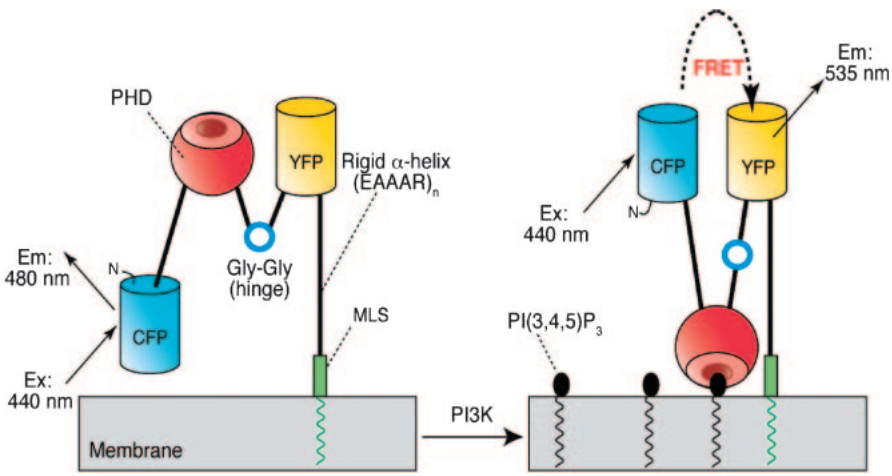

Fig. 4. Principle of the fluorescent indicator for PIP_3, named "Fllip."

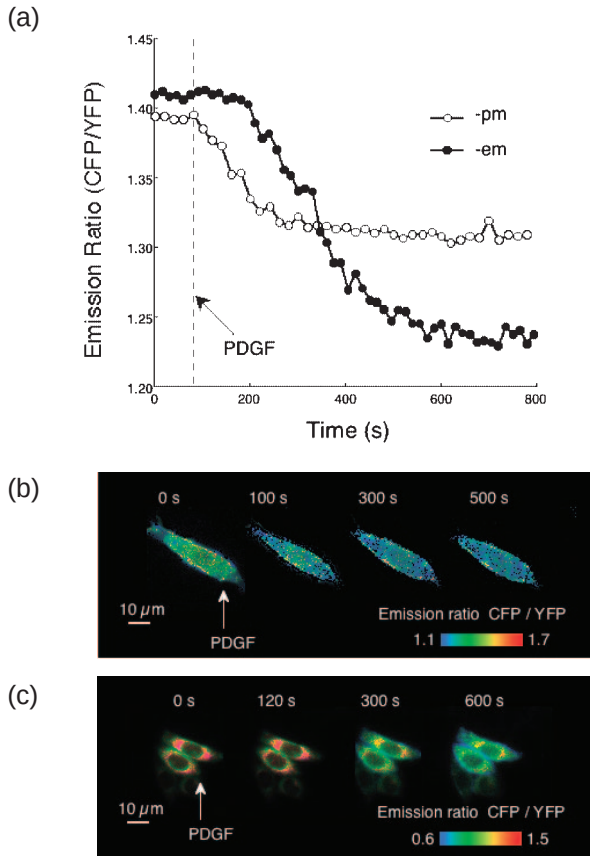

Fig. 5. Imaging PIP_3 dynamics at subcellular membranes. (**a**) Time courses of the emission ratio of Fllip-em (closed circle) and Fllip-pm (open circle) upon stimulation with platelet-derived growth factor (PDGF). The cells were stimulated with 50 ng mL^{-1} PDGF at the time shown with a broken line. (**b**) Pseudocolor images of the emission ratio of CFP to YFP before and 100, 300, and 500 s after the addition of 50 ng mL^{-1} PDGF, obtained from the CHO-PDGFR cells expressing Fllip-pm. (**c**) Pseudocolor images of the CFP/YFP emission ratio before and 120, 300, and 600 s after the addition of 50 ng mL^{-1} PDGF, obtained from the CHO-PDGFR cells expressing Fllip-em.

messengers. Fllip has two key sections, the PHD and MLS (Fig. 4). The PHD is important for the selectivity of Fllip for PIP_3. If the PHD is replaced with domains that bind lipid messengers other than PIP_3, such as diacylglycerol, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 4,5-bisphosphate, this allows us to change the selectivity of the indicator. Also, by connecting each specific MLS, it should be possible to direct the indicator not only to the plasma membrane and endomembranes but also to other organelle membranes, such as nuclear inner membrane and outer membrane of mitochondria. Next, I describe fluorescent indicators for diacylglycerol that visualize its dynamics at subcellular membranes in living cells.

3.2 Diacylglycerol. Diacylglycerol (DAG) is a lipid second messenger that recruits signaling proteins, such as protein kinase C isoforms, Ras guanyl nucleotide-releasing proteins, and chimaerins, and hence activates them at the membrane regions where DAG is generated (Fig. 3b).[21] Through the spatially regulated activation of the signaling proteins, DAG controls a broad spectrum of cellular functions, such as the regulation of vesicle transport from the Golgi apparatus to the plasma membrane and that of cell proliferation. To understand how DAG regulates each of the cellular functions, it is thus critical to find out where, when and how DAG increases and/or decreases in living cells.

We developed FRET-based indicators for DAG using a cysteine-rich domain (CRD) from protein kinase Cβ that specifically binds with DAG.[22] The DAG indicator emits FRET signals upon binding with DAG at cellular membranes. We named the DAG indicator "Daglas" (a fluorescent indicator for DAG that is locally generated at subcellular membranes).[23] We tethered Daglas to the plasma membrane, endomembranes, and mitochondrial outer membranes. The DAG indicators were used to clarify how DAG concentrations increase and/or decrease in discrete subcellular membranes in the absence and presence of the acute stimulation. We found that DAG is generated not only at the plasma membrane but also at organelle membranes, such as the endomembranes and mitochondrial outer membranes (Fig. 6). Previous studies have shown that DAG-binding proteins, such as protein kinase D[24] and RasGRP1,[25] are activated at the endomembranes to regulate cellular functions, such as vesicular transport and cell

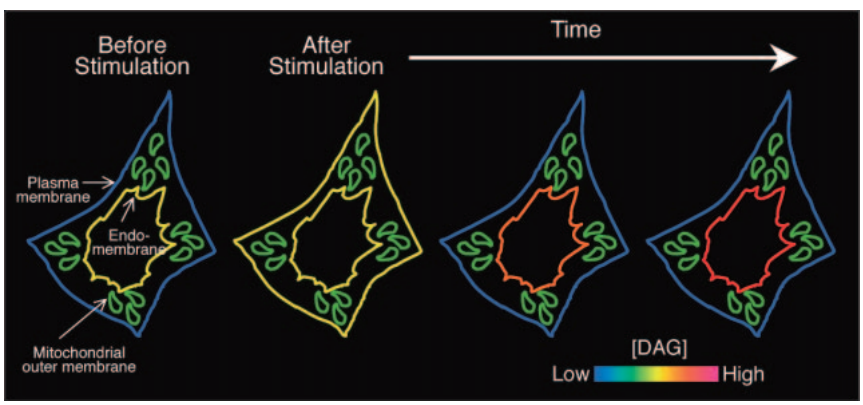

Fig. 6. Schematic diagram of DAG dynamics at discrete subcellular membranes before and after the physiologic stimulation. Changes in DAG concentrations are depicted by pseudocolor changes at the plasma membrane, endomembranes, and mitochondrial outer membranes.

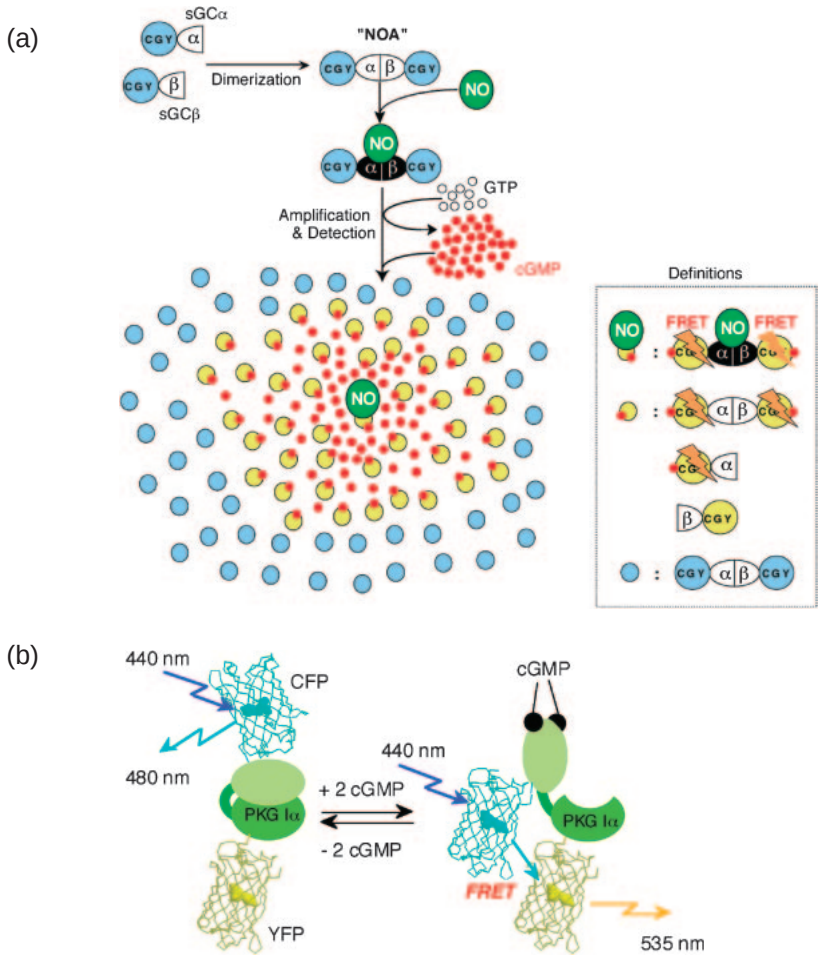

Fig. 7. An amplifier-coupled fluorescent indicator NOA for visualizing NO in single living cells. (**a**) Principle of NOA. sGCα-CGY and sGCβ-CGY are spontaneously associated to form a matured heterodimer, that is, NOA-1. NOA binds with NO and generates cGMP at the rate of 3000–6000 molecules min^{-1}. Thus, generated cGMP binds to the CGY domain in NOA make NOA emit a FRET signal. Approximately 99.9% of cGMP molecules thus generated diffuse and bind to NO-free NOA. As a result, even a single NO molecule can trigger a large number of NOA to emit FRET signals. Even if sGCα-CGY and sGCβ-CGY exist as monomers, the monomers also emit FRET signals upon binding with generated cGMP. (**b**) Principle of a cGMP indicator, CGY. CGY contains major part of cGMP-dependent protein kinase (PKGI$\alpha_{\Delta 1-47}$) having two cGMP-binding sites and thus binds with two molecules of cGMP. The CGY bound with two cGMP molecules emits a fluorescence signal based on FRET from CFP to YFP, which are respectively attached at N and C termini of PKGI$\alpha_{\Delta 1-47}$ in CGY.

differentiation. However, the key dynamics of DAG that controls activities of the signaling proteins have remained unknown at the endomembranes. Our measurement of the dynamics of DAG provides a molecular basis for the DAG-dependent activation of the signaling proteins at the endomembranes. We also found that tonic DAG is continuously generated at the mitochondrial outer membranes (Fig. 6). The mitochondrial tonic DAG appears to recruit DAG-binding proteins to the mitochondrial outer membranes and plays a critical role in the regulation of cellular functions unique to mitochondria.

4. Fluorescent Indicators for a Gaseous Second Messenger, Nitric Oxide

4.1 Fluorescent Molecular Indicator for Nitric Oxide. Since the finding of nitric oxide (NO) as endothelium-derived relaxing factor in 1987, it has become well established that NO is a ubiquitous messenger not only for vascular homeostasis but also for neurotransmission and immune system.[26] For the detection of NO, several methods have been devised so far. However, clear methods are not currently available to visualize NO dynamics in single living cells that have enough sensitivity (nanomolar) and spatial resolution (sub-micrometer). We recently developed a genetically encoded fluorescent indicator for NO that reversibly detects NO with a high sensitivity (detection limit of 0.1 nM) (Fig. 7a), and visualized the nanomolar dynamics of NO in single living cells.[27]

NO binds to its receptor protein, soluble guanylate cyclase (sGC), which thereby mediates many of the signaling functions of NO. sGC is a heterodimer consisting of α- and β-subunits (sGCα and sGCβ), and sGCβ contains an N-terminal heme-binding region, to which NO reversibly binds. Both sGCα and sGCβ subunits share a C-terminal cyclase domain. After NO binds to the heme group, cyclase activity is stimulated up to 400-fold, resulting in the repeated conversion of guanosine triphosphate (GTP) to guanosine 3′,5′-cyclic mono-

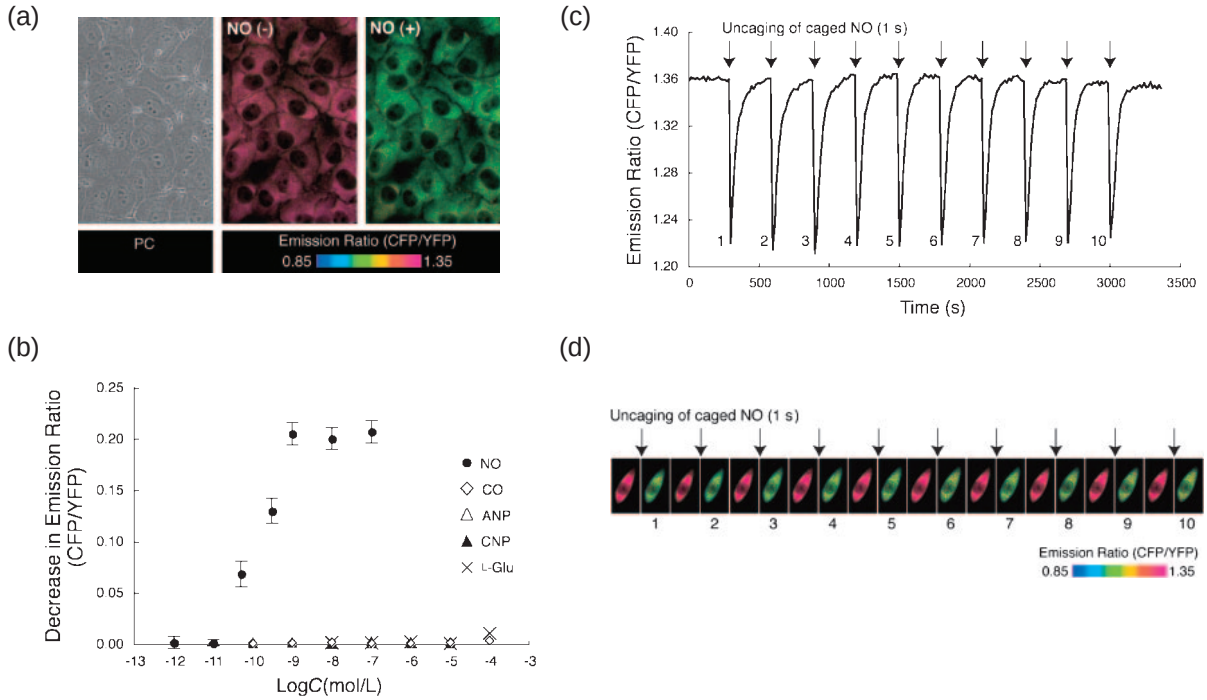

Fig. 8. A cell-based fluorescent indicator Piccell for NO. (**a**) A phase contrast image of Piccell (left) and pseudocolor image of the CFP/YFP emission ratio of Piccell in the absence (center) or presence (right) of 10 nM NO. (**b**) Dose-response relationships of Piccell for NO, carbon monoxide (CO), atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), and L-glutamate (L-Glu). (**c**) Every time NO was generated for 1 s by photolysis of the caged NO, Piccell exhibited a sharp FRET response. The uncaging of the caged NO was repeated consecutive 10 times. The result confirms the reversibility and reproducibility of Piccell. (**d**) Pseudocolor images of the CFP/YFP emission ratio of Piccell before and after repeated uncaging of the caged NO in Fig. 8c. The numbers 1–10 in this figure correspond to the repeated uncaging of the caged NO in Fig. 8c.

phosphate (cGMP). To develop a genetically encoded NO indicator, we designed two chimera proteins. One was sGCα which was connected to a genetically encoded fluorescent indicator for cGMP, which we have previously developed and named CGY,[28] and the other was sGCβ connected to CGY. CGY emits FRET signals upon binding cGMP (Fig. 7b). When expressed in living cells, sGCα-CGY and sGCβ-CGY are spontaneously associated to form a matured heterodimer as well as sGCα and sGCβ (Fig. 7a). This heterodimer of sGCα-CGY and sGCβ-CGY binds NO, generates cGMP at the rate of 3000–6000 molecules min^{-1} and then emits a FRET signal from the CGY domain upon binding four molecules of the generated cGMP. More interestingly, the residual cGMP molecules diffuse and bind to the CGY domain within NO-free heterodimers of sGCα-CGY and sGCβ-CGY surrounding the NO-bound heterodimer. Thus, a single NO molecule provokes FRET signals from a large number of the NO-free heterodimers through the residual cGMP molecules (Fig. 7a). Even if sGCα-CGY and sGCβ-CGY exist as monomers due to their unbalanced expression in living cells, the monomers emit FRET signals upon binding the residual cGMP as well as the heterodimers of sGCα-CGY and sGCβ-CGY. We named the heterodimer of sGCα-CGY and sGCβ-CGY "NOA" (a fluorescent indicator for NO with a signal amplifier).[27]

Due to its amplifier-coupled mechanism, NOA exhibits a high sensitivity to nanomolar concentrations of NO. Organic fluorescent dyes have been reported for NO detection, such as diamino-fluoresceins (DAFs)[4] and diamino-cyanines (DACs).[29] NOA appears to have approximately 10000-fold higher sensitivity than the organic dyes in living cells. Furthermore, in contrast to the previous organic dyes, NOA exhibits the reversible response to NO in living cells, because the reversible binding of NO to NOA and endogenous phosphodiesterases, which immediately hydrolyze the generated cGMP molecules, guarantees the reversible response of NOA to NO in living cells. In addition, the present NOA detects biologically important NO radicals, whereas the previous organic dyes could not be used to detect the NO radical but could be used to detect its metabolite, NO^+. The superiority of NOA to the previous organic dyes in terms of sensitivity and reversibility, in particular, contributes to understanding biology of the nanomolar concentrations of NO.

4.2 Cell-Based Fluorescent Indicator for Nitric Oxide. Because NO is a small uncharged free radical, it acts as a diffusible intercellular messenger, like paracrine factors including cytokines and neurotransmitters.[26] It is thus of essential importance to analyze the spatiotemporal dynamics of NO release from the cell for the understanding of biological processes of NO. We developed a novel cell-based fluorescent indicator for NO, named Piccell.[30]

For the construction of Piccell, we permanently expressed our fluorescent cGMP indicator, CGY (Fig. 7b), in PK15 cells that endogenously express both α- and β-subunits of sGC (Fig. 8a). Piccell thus emits FRET signals in response to NO through the generated cGMP (Fig. 8a). As to the sensitivity of Piccell, because a single NO molecule stimulates the gener-

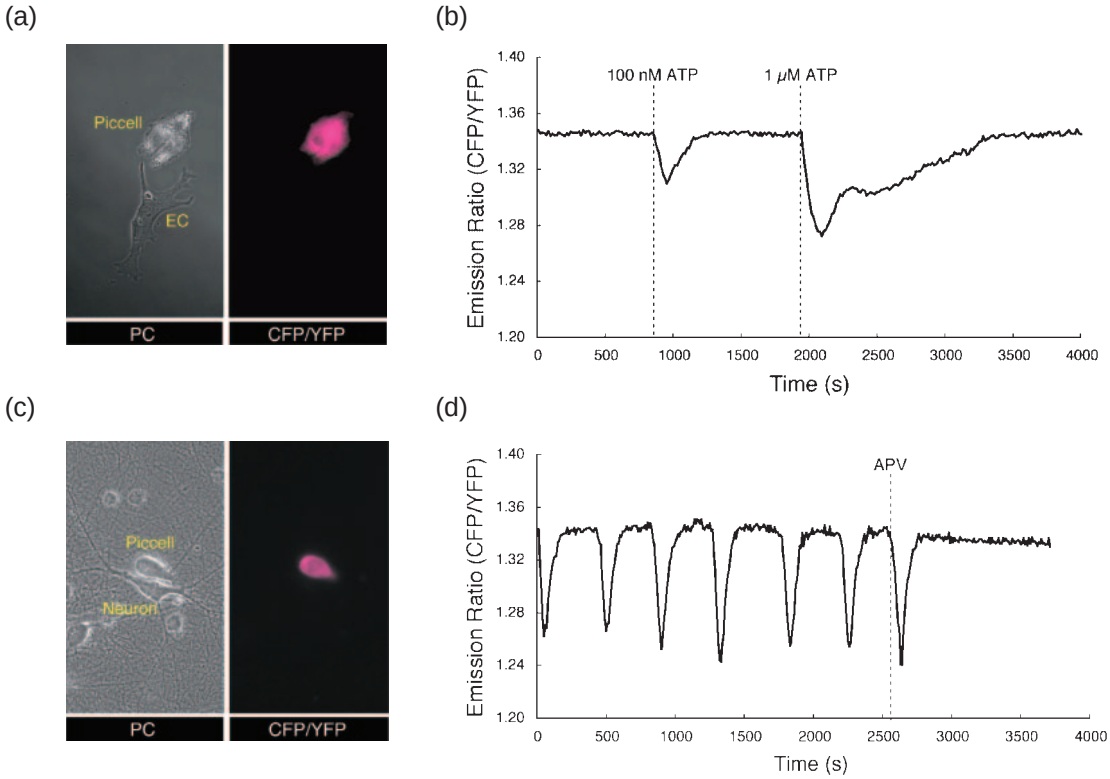

Fig. 9. Piccell visualizes the NO release from living cells. (**a**) A phase contrast image (left) and pseudocolor image of the CFP/YFP emission ratio (right) of a cocultured vascular endothelial cell and Piccell. (**b**) Time course of the FRET response of Piccell for 100 nM and subsequent 1 μM ATP stimulations, when Piccell was cocultured with the endothelial cell. (**c**) A phase contrast image (left) and pseudocolor image of the CFP/YFP emission ratio (right) of cocultured hippocampal neurons and Piccell. (**d**) Time course of the FRET response of Piccell cocultured with hippocampal neurons. The oscillatory FRET response of Piccell cocultured with neurons was blocked with an inhibitor of NMDA receptor, 2-amino-5-phosphonovaleric acid (APV).

ation of cGMP at the rate of ≈6000 molecules min^{-1}, FRET signals from a large number of the cGMP indicators in the cell-based indicator occur. This provides a molecular basis for the outstanding sensitivity of Piccell that detects picomolar concentrations of NO (detection limit of 20 pM) (Fig. 8b). We also confirmed the selectivity, reversibility, and reproducibility of Piccell (Figs. 8b–8d).

We cocultured Piccell with vascular endothelial cells (Fig. 9a). Piccell adjacent to an endothelial cell could be used to visualize the ligand-dependent NO release from the endothelial cell (Fig. 9b). We next cocultured Piccell with hippocampal neurons to visualize the picomolar dynamics of neuronal NO (Fig. 9c). It was shown with Piccell that hippocampal neurons release picomolar concentrations of NO spontaneously and periodically, that is, NO oscillation (Fig. 9d). The averaged peak response of Piccell indicates that 100 pM of NO is periodically released from neurons. We also showed that the oscillatory release of picomolar concentrations of neuronal NO is controlled by *N*-methyl-D-aspartic acid (NMDA) receptors, which are activated by spontaneous neurotransmission between neurons (Fig. 9d). There is mounting evidence that NO is a key factor that regulates neuronal functions.[31,32] However, how much and how NO is generated and released from neurons have remained unclear. The oscillatory release of picomolar concentrations of NO from neurons, which we have found using Piccell, appears to be fundamental to the regulation of neuronal functions, such as neurotransmission, learning and memory. Piccell provides a powerful tool to uncover dynamics of NO at the picomolar level, which regulates a wide range of cell functions in biological systems.

5. Conclusion

I reviewed here several examples among our genetically encoded fluorescent indicators for visualizing a wide variety of molecular process in living cells. The theory of FRET was developed by Förster in 1940's.[33] Since the end of 1970's, FRET has frequently been used for biochemical analysis.[34] Mitra et al.[35] and Heim and Tsien[36] have reported fluorescent protein-based FRET technology in 1996. Since then, researchers using genetic engineering techniques have started using the FRET technology.[37] For example, Romoser et al.[38] and Miyawaki et al.[39] have developed their own FRET-based indicators in 1997 to visualize the dynamics of a second messenger Ca^{2+} in living cells.

My motivation in the development of fluorescent indicators has not been limited to the observations of the spatiotemporal dynamics of molecular processes in living cells. The present indicators provide a powerful tool to examine in more detail how signaling processes are regulated if they are used together with pharmacological or molecular biological techniques that inhibit particular signaling processes in the cell. We actually showed how the spatiotemporal generation of second messen-

gers and the activation of protein kinases, observed with our indicators, are regulated in the cell. Our fluorescent indicators has provided biologically significant information, which is unobtainable with previous methods, and are thus advantageous for basic research to examine the molecular events in single living cells with high spatial and temporal resolution.

Recently, a wide variety of fluorescent proteins ranging from blue to red have been cloned from living subjects in the sea.[37,40] Fluorescent protein pairs having larger spectral overlaps have also been identified compared with the pair of CFP and YFP used for our fluorescent indicators. These color variants of fluorescent proteins, which are more appropriate for FRET than CFP and YFP, may improve the FRET efficiencies of our genetically encoded fluorescent indicators, making the indicators easier to use for a broad range of biological applications.

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