Optical Probes for Molecular Processes in Live Cells

Yoshio Umezawa

Department of Chemistry, School of Science, University of Tokyo, Tokyo 113-0033, Japan; email: umezawa@chem.s.u-tokyo.ac.jp

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Abstract

In this review, I summarize the development over the past several years of fluorescent and/or bioluminescent indicators to pinpoint cellular processes in living cells. These processes involve second messengers, protein phosphorylations, protein-protein interactions, protein-ligand interactions, nuclear receptor–coregulator interactions, nucleocytoplasmic trafficking of functional proteins, and protein localization.

1. PROBING CELLULAR SIGNALING PATHWAYS IN LIVING CELLS

Organic fluorescent probe molecules have been developed for nondestructive analysis of chemical processes in living cells, including ions and small molecules such as Ca^{2+} (1), NO (2), Mg^{2+} (3), and Zn^{2+} (4). In addition, green fluorescent protein (GFP) and its analogs have been used to probe proteins to determine their structural and locational changes after genetically labeling them to proteins of interest (5).

Many intracellular chemical processes and cellular signaling processes are still studied essentially by destructive analysis. Such methods disrupt hundreds of thousands of cells prior to separation, purification, and detection of intracellular components. It is necessary, therefore, to develop methods for direct nondestructive analysis of cellular signaling steps in live cells.

Intercellular signaling substances include neurotransmitters, which are cytokines and hormones functioning in the nerve, immune, and endocrine systems. These substances bind either to ion-channel-, kinase-, or G protein–coupled membrane receptor proteins and trigger the respective downstream intracellular signaling processes. Intracellular signaling can be monitored in vivo in living cells (6) by genetically encoded intracellular fluorescent and bioluminescent probes or indicators. Scientists have reported a number of these probes for visualizing cellular signaling. The probes include second messengers such as Ca^{2+} (7), camp (8), nitric acid (NO) (9), inositol 1,4,5-trisphosphate (IP_3) (10, 11), cyclic guanosine 3′,5′-monophosphate (cGMP) (12), and phosphatidylinositol-3,4,5-trisphosphate (13), protein phosphorylation (14–16), protein-protein interactions (17–21), and protein localizations in organelles (22–26). These probes are useful not only for fundamental biological studies, but also for the assay and screening of possible pharmaceutical or toxic chemicals that inhibit or facilitate cellular signaling pathways.

2. SECOND MESSENGERS

2.1. Nitric Oxide

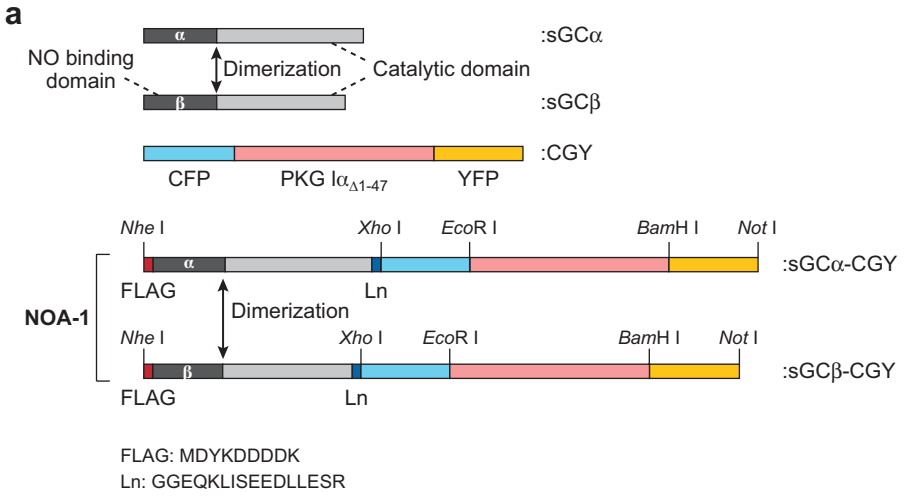
Nitric oxide (NO) is a small uncharged free radical that is involved in diverse physiological and pathophysiological mechanisms. NO is generated by three isoforms (endothelial, neuronal, and inducible) of NO synthase (NOS). When generated in vascular endothelial cells, NO plays a key role in vascular tone regulation. An amplifier-coupled fluorescent indicator for NO was developed (9) to visualize physiological nanomolar dynamics of NO in living cells (detection limit of 0.1 nM). Earlier, a cGMP fluorescent indicator, cyan fluorescent protein/protein kinase G/yellow fluorescent protein (known as CGY), was developed, which was combined with soluble guanylate cyclase (sGC) for the amplified detection of NO in living cells (9). This amplifier-coupled fluorescent indicator was named NOA-1. NOA-1 binds with single NO molecules and generates a large number of cGMPs in single living cells. The increased amount of cGMP in situ is detected by the cGMP fluorescence resonance energy transfer (FRET) sensor built into NOA-1. NOA-1 unbound to cGMP does

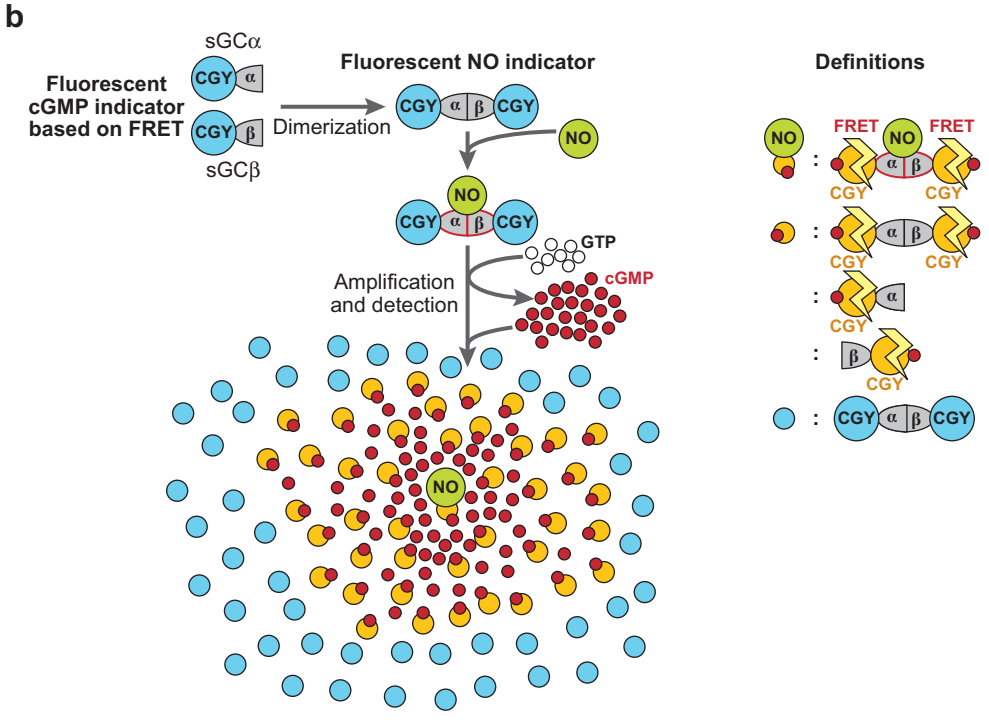
not emit the FRET signal. The vascular endothelial cell stably generates 1 nM of the basal NO. This genetically encoded high-sensitivity indicator revealed that approximately 1 nM of NO, which is enough to relax blood vessels, is generated in vascular endothelial cells even in the absence of shear stress. The nanomolar range of basal endothelial NO thus revealed appears to be fundamental to vascular homeostasis (9) (**Figure 1**).

We report a novel cell-based indicator that is able to visualize picomolar dynamics of NO release from living cells. Cells from a pig kidney–derived cell line (PK15) endogenously express sGC, which is a receptor protein for the selective recognition of NO. Binding of NO by sGC causes the amplified generation of cGMP. To make the PK15 cells into NO indicators, the cells are transfected with a plasmid vector encoding a fluorescent indicator for cGMP, and FRET is recorded at 480 $\pm$ 15 and 535 $\pm$ 12.5 nm upon excitation of the cells at 440 $\pm$ 10 nm. The cell-based indicator exhibits exceptional sensitivity (detection limit of 20 pM), selectivity, reversibility, and reproducibility. The outstanding sensitivity of the present indicator has led us to uncover an oscillatory release of picomolar concentrations of NO from hippocampal neurons. We present evidence that Ca^{2+} oscillations in hippocampal neurons underlie the oscillatory NO release from the neurons during neurotransmission. We have also succeeded in visualizing the extent of diffusing NO from single vascular endothelial cells. The present cell-based indicator provides a powerful tool for uncovering picomolar dynamics of NO that regulate a wide range of cell functions in biological systems (27).

2.2. Phosphatidylinositol-3,4,5-Trisphosphate

Phosphatidylinositol-3,4,5-trisphosphate (PIP_3) regulates diverse cellular functions, including cell proliferation and apoptosis, and has roles in the progression of diabetes and cancer. However, little is known about its production. Fluorescent indicators for PIP_3 have been developed based on FRET (13). These novel PIP_3 indicators are composed of two distinctly colored mutants of GFP and a PIP_3-binding domain. The PIP_3 level was observed by dual-emission ratio imaging, thereby allowing stable observation without the problem of artifacts. Furthermore, these indicators were fused with localization sequences to direct them to the plasma membrane or endomembranes, allowing localized analysis of PIP_3 concentrations. Using these fluorescent indicators, we analyzed the spatiotemporal regulation of the PIP_3 production in single living cells. To examine PIP_3 dynamics, a pleckstrin homology (PH) domain from GRP1 was used, which selectively binds PIP_3, fused between cyan and yellow fluorescent protein (CFP and YFP, respectively) variants through rigid α-helical linkers, 12 of which consist of repeated EAAAR sequences. Within one of the rigid linkers, a single diglycine motif was introduced as a hinge. We then tethered the chimeric indicator protein to the membrane by fusing it with a membrane localization sequence through the rigid α-helical linker. Thus, after PI(3)K activation, the PH domain binds to PIP_3 and a significant conformational change of the indicator protein occurs through the flexible diglycine motif introduced into the rigid α-helical linker. This "flip-flop-type" conformational change of the indicator protein changes





intramolecular FRET from CFP to YFP, allowing detection of PIP_3 dynamics at the membrane. We named this indicator fllip (fluorescent indicator for a lipid second messenger that can be tailor made).

The developed fllip allows a spatiotemporal examination of PIP_3 production in single living cells. After ligand stimulation, PIP_3 levels increased to a larger extent at the endomembranes (i.e., the endoplasmic reticulum and the Golgi) than at the plasma membrane. This increase was found to originate from in situ production at the endomembranes, a process stimulated directly by receptor tyrosine kinases endocytosed from the plasma membrane to the endomembranes. The demonstration of PIP_3 production through receptor endocytosis addresses a long-standing question about how signaling pathways downstream of PIP_3 are activated at intracellular compartments remote from the plasma membrane (13) (**Figure 2**).

We described fluorescent indicators for a lipid second messenger, diacylglycerol (DAG), which allow the localized analysis of DAG dynamics at subcellular membranes. We have shown that DAG concentrations increase and/or decrease at not only the plasma membrane, but also at organelle membranes such as endomembranes and mitochondrial outer membranes (13).

3. PROTEIN PHOSPHORYLATIONS

Protein phosphorylation by intracellular kinases plays one of the most pivotal roles in signaling pathways within cells. The kinase proteins catalyze transfer of the phosphate of ATP and phosphorylation of the hydroxy groups of serines, threonines, and/or tyrosines on the substrate proteins. Upon phosphorylation, the substrate proteins undergo conformational changes caused by the negative charges of the phosphates, which subsequently trigger their enzymatic activation and interaction with their respective target proteins. To reveal the biological issues related to the kinase proteins, electrophoresis, immunocytochemistry, and in vitro kinase assay have been used. However, these conventional methods do not provide sufficient information about the spatial and temporal dynamics of signal transduction based on protein phosphorylation and dephosphorylation in living cells. To overcome the limitations

Figure 1

An amplifier-coupled fluorescent indicator for visualizing nitric oxide (NO) in single living cells. (*a*) Schematic representations of domain structures of soluble guanylate cyclase (sGC), CGY, sGC-CGY, and sGC-CGY. The amino acid sequence of FLAG tag and linker (Ln) is shown at the bottom. The heterodimer of sGC-CGY and sGC-CGY has been named NOA-1. (*b*) Principle of the NO indicator NOA-1. sGC-CGY and sGC-CGY are spontaneously associated to form a matured heterodimer, NOA-1. NOA-1 binds with NO and generates cyclic guanosine 3′,5′-monophosphate (cGMP) at the rate of 3000–6000 molecules/min. Thus, generated cGMP binds to the CGY domain in NOA-1 and causes NOA-1 to emit a fluorescence resonance energy transfer (FRET) signal. About 99.9% of cGMP molecules is thus generated diffusely and is bound to NO-free NOA-1. As a result, even a single NO molecule can trigger a large amount of NOA-1 to emit FRET signals. Even if sGC-CGY and sGC-CGY exist as monomers, the monomers also emit FRET signals upon binding with generated cGMP. Abbreviations: GFP, green fluorescent protein; PKG, protein kinase G.

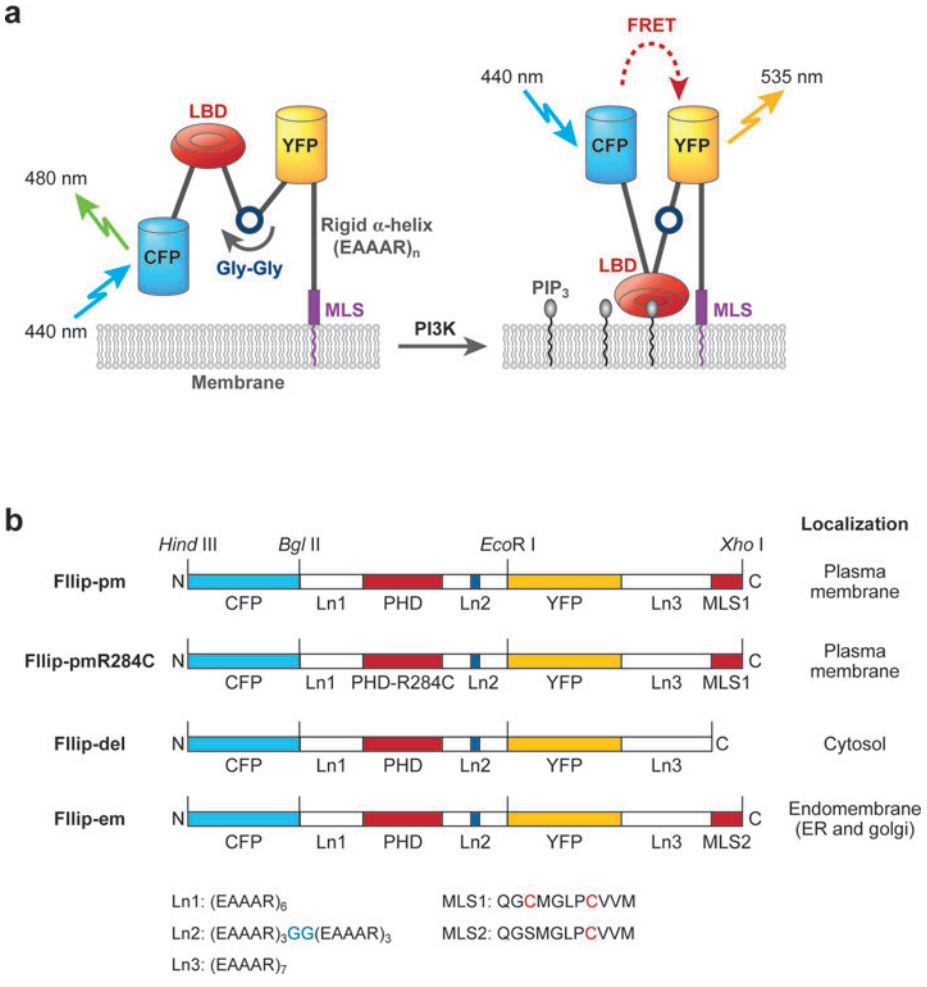


Figure 2

Fluorescent indicators for PtdInsP3 in single living cells. (*a*) Principle of fluorescent indicator for a lipid second messenger that can be tailor made (fflip) for visualizing PtdInsP3. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are different-colored mutants of green fluorescent protein with mammalian codons and additional mutations. After binding of PtdInsP3 to the pleckstrin homology (PH) domain within fflip, a flip-flop-type conformational change occurs, changing the efficiency of fluorescence resonance energy transfer from CFP to YFP. (*b*) Schematic representations of domain structures within the present fflips. The PH domain is derived from human GRP1 (amino acids 261–382) and selectively binds to PtdInsP3. PH domain R284C is a mutant PH domain, in which Arg 284 is replaced with cysteine, abolishing binding to PtdInsP3. Abbreviations: ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; Ln1, Ln2, and Ln3, linkers (the amino-acid sequences of which are shown at bottom); MLS1 and MLS2, membrane localization sequences for the plasma membrane and endomembranes, respectively, the amino-acid sequences of which are shown at bottom.

of investigating kinase signaling, genetically encoded fluorescent indicators have been developed for visualizing protein phosphorylation in living cells (14).

Besides traditional genomic pathways of sex steroid receptors in the nucleus, the extranuclear nongenomic pathways of these receptors have also been shown to strongly relate to many biological consequences, including vascular protection and cell proliferation (28–30). These nongenomic pathways are rapidly mediated through several critical protein kinases. A nonreceptor protein tyrosine kinase, Src, is known to be activated immediately after a steroid stimulation (31, 32). The activated Src phosphorylates various substrate proteins, such as Shc, that finally induce ERK-dependent transcription (33, 34). To determine how the Src activity is nongenomically regulated by steroid receptors in single living cells, we developed a fluorescent indicator for Src kinase activity and named it Srcus. This indicator can monitor substrate phosphorylation by activated endogenous Src as a FRET response in single living cells.

Based on the fluorescence imaging with the present fluorescent indicators, we demonstrated that E2-induced Src activation takes place in not only plasma- but also endomembranes. This was ascribed to the existence of epidermal growth factor (EGF) and occurrence of EGF receptor– (EGFR-)involved endocytosis of estrogen receptor (ER) together with Src. EGFR, ER, and Src were found to form a ternary complex upon E2 stimulation. The cell growth of breast cancer–derived MCF-7 cells increased markedly through the above EGF-involved estrogen-signaling process. In contrast to estrogen-activated Src signaling, the male steroid hormone, 5α-dihydroxytestosterone (DHT), was found to activate Src only in the plasma membrane free from the interaction of EGFR with androgen receptor (AR). The cell growth occurred only moderately as a result. The spatial difference in Src activation between E2 and DHT may be responsible for the different rates of MCF-7 cell growth between E2 and DHT (39) (**Figure 3**).

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. To study the spatiotemporal dynamics of protein phosphorylation by activated ERK in living cells, we have developed genetically encoded fluorescent indicators for ERK (39). The present indicators change their conformation upon protein phosphorylation by activated ERK and then emit fluorescence signals based on FRET. We visualized the cytosolic and nuclear activity of ERK using the present indicators. We thus found that the activation duration of ERK is considerably different between the cytosol and nucleus in living cells. The subcellular differences in ERK activity may be fundamental to the regulation of cell functions by ERK (**Figure 4**).

Cholesterol-enriched nanodomains called lipid rafts are thought to act as a platform for protein signaling in cells, but the physiologic significance of lipid rafts in cells and tissues is still unknown (35, 36). The main point of the present work is to show the physiologic significance of kinase activity in lipid rafts. Src family kinases (SFKs) are known to be distributed throughout the cell membranes and to regulate many biological processes (37, 38). To locate with high resolution where SFK activation occurs in the cell membranes, we developed a genetically encoded transmembrane fluorescent indicator for detecting the SFK activation at the cell membranes, and named it TM-Srcus (14, 39). TM-Srcus can monitor the substrate

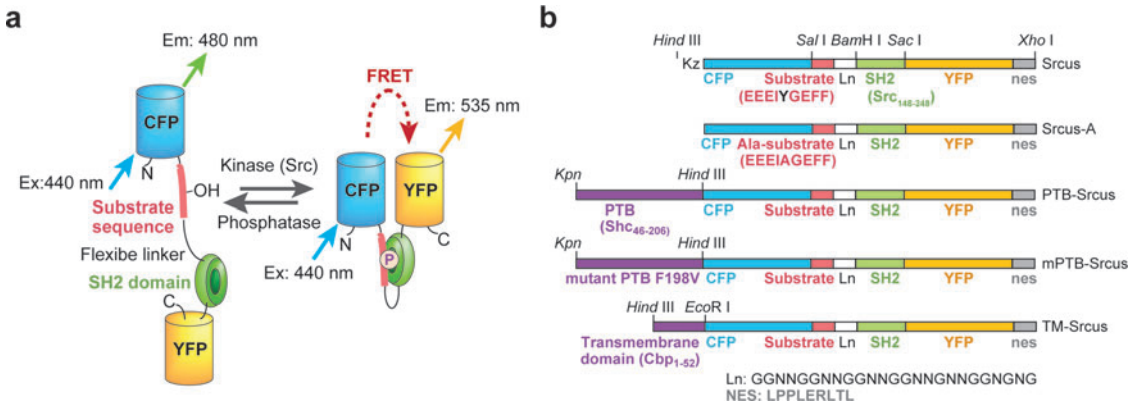


Figure 3

Fluorescent indicators visualize rapid Src signaling stimulated by sex steroids. (*a*) Principle of Srcus for monitoring Src activation. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are different-colored mutants of green fluorescent protein (GFP). Upon phosphorylation of the substrate sequence within Srcus by Src, the adjacent tyrosine phosphorylation recognition (SH2) domain binds with the phosphorylated substrate sequence, which increases the efficiency of fluorescence resonance energy transfer between the GFP mutants within Srcus. (*b*) Schematic representations of domain structures of Srcuses. Restriction sites are also shown with the constructs. A Kozak sequence (*Kz*) allows optimal translation initiation in mammalian cells. The amino acid sequence of the substrate sequence is EEEIYGEFF, which is preferentially phosphorylated by Src. The SH2 domain is derived from Src-(148–248). The phosphotyrosine binding (PTB) domain is derived from Shc-(46–206). The transmembrane domain is from Cbp-(1–52). In the mutant PTB F198V domain, an amino acid residue at position 198 of Shc-(46–206) is mutated from phenylalanine to valine. Abbreviations: FRET, fluorescence resonance energy transfer; Ln, flexible linker sequence (GGNNGGNNGGNNGGNNGGNNGGNGNG); NES, nuclear-export-signal sequence derived from the human immunodeficiency virus protein Rev.

phosphorylation by activated SFKs as the decrease in the CFP/YFP emission ratio through FRET. The total internal reflection fluorescence imaging of the SFK activation on the plasma membrane by TM-Srcus showed that SFK activation takes place in lipid rafts.

Based on this finding, we developed a lipid raft–targeted SFK inhibitory fusion protein (LRT-SIFP) to inhibit SFK activity in lipid rafts. The LRT-SIFP contains the peptide inhibitor of SFK and the targeting sequence for localizing the SIFP to lipid rafts. The significance of the subcellular locations of kinase activity has hardly been studied by conventional inhibition methods such as small interfering RNA and chemical or peptide inhibitors, which inhibit kinase activity in cells. The present LRT-SIFP highlighted the importance of SFK activation in lipid rafts in the function of breast cancer cells. Although it is highly potent, the previously developed peptide inhibitor of SFK does not affect cell functions of MCF-7 cells derived from human breast cancer. In contrast to this conventional peptide inhibitor, the present LRT-SIFP inhibits cell adhesion and cell cycle progression of MCF-7 and MDA-MB231 cells. In addition, these inhibitory effects of LRT-SIFP on cell functions are

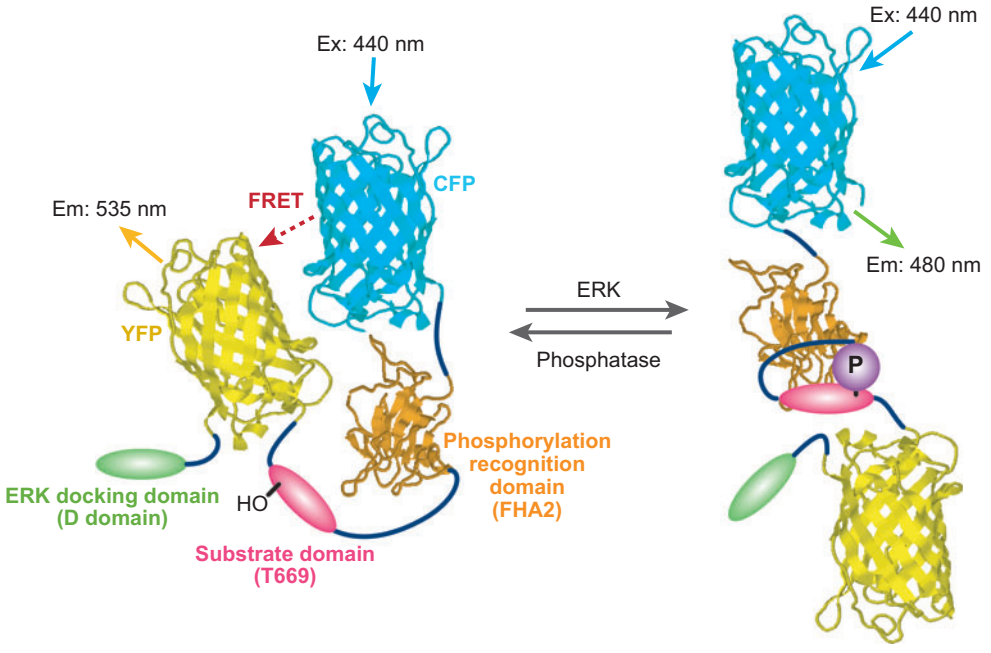


Figure 4

Design of the present fluorescent indicator to visualize protein phosphorylation by extracellular signal–related kinase (ERK), Erkus. Erkus consists of CFP, FHA2 domain, substrate domain, yellow fluorescent protein (YFP), and D domain. Cyan fluorescent protein (CFP) and YFP are different-colored mutants of *Aequorea victoria* fluorescent proteins. Upon phosphorylation, the substrate domain binds with the FHA2 domain, resulting in the decrease of fluorescence resonance energy transfer (FRET) efficiency from CFP to YFP.

specific for tumor cell lines. The lipid raft–specific knockdown of SFK activity would potentially be useful for selective cancer therapy to prevent tumorigenesis and metastasis of breast cancer cells (**Figure 5**).

4. PROTEIN-PROTEIN INTERACTIONS

Protein-protein interactions play pivotal roles in many chemical processes in living cells, yet they have been among the most difficult aspects of molecular and cellular biology to be studied. Monitoring protein-protein interactions in living cells is important for screening and assaying chemicals that increase or inhibit cellular signaling processes. To promote a greater understanding of the chemical processes, several methods have been developed for detecting protein-protein interactions. Available information about protein-protein interactions was obtained mostly via biochemical methods, but these methods required destructive analysis, which did not provide us with live-cell dynamics. The yeast two-hybrid system (40, 41) and the mammalian two-hybrid system (42, 43) use a "bait" protein fused to a DNA binding domain with a nuclear localization signal (NLS) in order to find their "prey" protein connected

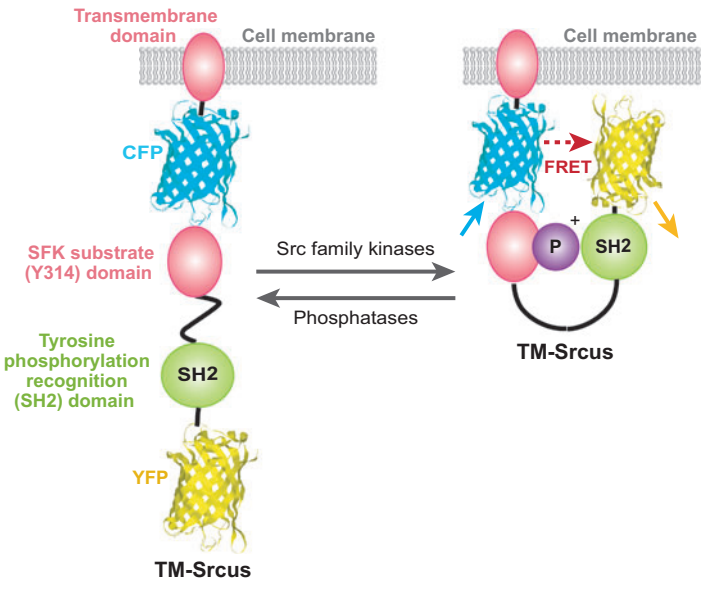


Figure 5

TM-Srcus, a fluorescent indicator for detecting Src family kinase (SFK) activation in cell membranes. The principle of TM-Srcus for visualizing SFK activation in cell membranes. On SFK activation, a conformational change in TM-Srcus occurs as a result of the binding of a tyrosine phosphorylation recognition (SH2) domain to a phosphorylated SFK substrate (Y314) domain, which results in an intramolecular fluorescence resonance energy transfer response. Abbreviation: FRET, fluorescence resonance energy transfer.

to a transcription activation domain with an NLS. The interaction between bait and prey accumulates the transcription activation domain on a specific sequence of DNA located upstream of a reporter gene; thus, the reporter gene expression is transactivated. Although significant signals for detection are obtained with the two-hybrid systems, they are limited in that detectable protein-protein interactions occur only in the nucleus to transactivate the reporter gene (44–46).

The split ubiquitin system (47–49) for detecting an interaction between a membrane protein and a cytosolic protein also limits the detection of interactions between cytoplasm proteins or nuclear localizing proteins. Several methods have been reported as useful in overcoming these limitations, including the protein complementation system such as using firefly luciferase (50), and *Renilla* luciferase (20, 51). We previously proposed a novel concept, a protein reconstitution system based on protein splicing, for detecting protein-protein interactions (18, 19, 52) and protein localization in organelles (22, 24). Although the protein complementation system and the protein reconstitution system allow us to monitor interactions between cytoplasm and membrane-proximal proteins, they suffer from low-endpoint signals for the interactions due to the much lower activity of complemented or reconstituted split reporters compared with that of intact reporter proteins. Here we describe a protein splicing–based reporter gene assay to monitor protein-protein interactions

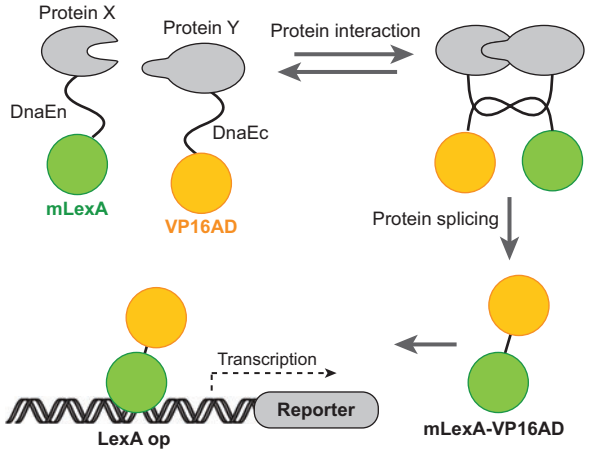


Figure 6

Principle for the intein-mediated reporter gene assay. DnaEn (amino acids 1–123) and DnaEc (amino acids 1–36) are connected with modified LexA (mLexA) (amino acids 1–229) and the transcription activation domain of a herpes simplex virus protein (VP16AD) (amino acids 411–456), respectively. Interested proteins X and Y are linked to the ends of DnaEn and DnaEc, respectively. Interaction between X and Y accelerates the folding of DnaEn and DnaEc, and protein splicing results. mLexA and VP16AD are linked together by a peptide bond to obtain a transcriptional activity.

in mammalian cells. Protein splicing is a posttranslational autocatalytic process in which an intein is excised with the concomitant ligation of the flanking exteins (53–56). An important property of the protein splicing is that the substitution of exteins for different peptides does not interfere with the splicing process (57, 58). We chose N- and C-terminal halves of an Ssp-DnaE intein as the protein-splicing elements, and modified LexA (mLexA) and a transcription activation domain of a herpes simplex virus protein (VP16AD) as the transcription factors. The present reporter gene assay allowed us to detect EGF-induced membrane-proximal Ras-Raf-1 interactions that could not be detected with the previous reporter gene assay, the two-hybrids method. In addition, the present reporter gene assay enabled us to obtain sufficient signals for the interactions that were not identified with the firefly luciferase complementation system (**Figure 6**).

We developed an approach for discriminating agonist and antagonist in a nongenomic steroid-signaling pathway using an association of AR with Src. We constructed a pair of genetically encoded indicators, where N- and C-terminal fragments of split firefly luciferase (FLuc) were fused to AR and Src, respectively. The proteins fused with AR and Src are localized in the cytoplasm and on the plasma membrane, respectively. Upon being activated with androgen, AR undergoes an intramolecular conformational change and binds with Src. The association causes the complementation of the split FLuc and recovery of FLuc activity. The resulting luminescence intensities were taken as a measure of the rapid hormonal activity of steroids in the nongenomic AR signaling (**Figure 7**) (24, 25, 59, 60).

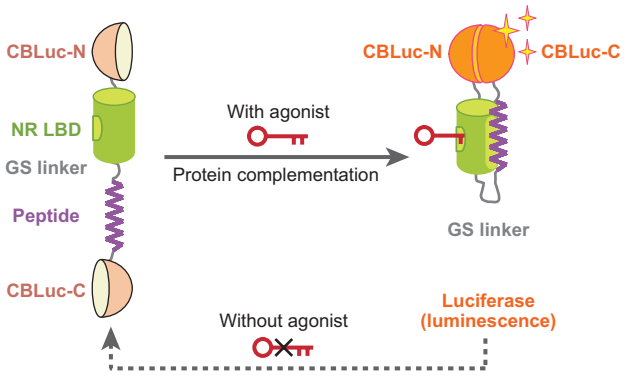


Figure 7

Schematic diagram showing the detection scheme of the single-molecule-format bioluminescent indicator based on an intramolecular complementation strategy of split click beetle luciferase (CBLuc) for monitoring bioactive small molecules. An agonist induces conformational change in the ligand binding domain of a nuclear receptor (NR LBD). It subsequently activates the association of NR LBD with the specific recognition peptide sequence. The association triggers the recovery of the CBLuc activities by an intramolecular complementation of split CBLuc. The recovered luciferase activities are taken as a measure of the androgenicity of ligands. On the other hand, removal of the agonist dissociates the complementation between NR LBD and the motif and cancels the developed CBLuc activities. The agonist was animated with a key, whereas the split CBLuc was drawn as a half-segmented ball. Abbreviations: CBLuc-N, N-terminal fragment of click beetle luciferase; CBLuc-C, C-terminal fragment of click beetle luciferase; GS linker, a flexible amino acid sequence consisting of glycines and serines.

5. PROTEIN-LIGAND INTERACTIONS

Click beetle luciferase (CBLuc) is insensitive to pH, temperature, and heavy metals, and emits a stable, highly tissue-transparent red light with luciferin in physiological circumstances. Thus, the luminescence signal is optimal for a bioanalytical index reporting the magnitude of a signal transduction of interest. We validated a single-molecule-format complementation system of split CBLuc to study signal-controlled protein-protein (peptide) interactions (61). First, we generated 10 pairs of N- and C-terminal fragments of CBLuc to examine whether a significant recovery of the activity occurs through the intramolecular complementation. The ligand binding domain of androgen receptor (AR LBD) was connected to a functional peptide sequence through a flexible linker. The fusion protein was then sandwiched between the dissected N- and C-terminal fragments of CBLuc. Androgen induces the association between AR LBD and a functional peptide and the subsequent complementation of N- and C-terminal fragments of split CBLuc inside the single-molecule-format probe, which restores the activities of CBLuc. Examination of the dissection sites of CBLuc revealed that the dissection positions next to the amino acids D412 and I439 admit a stable recovery of CBLuc activity through an intramolecular complementation.

Ligand-induced conformational changes of nuclear hormone receptors (NRs) are important initiators of various kinds of hormone signaling. However, little is known of the bioanalytical use of the hormone-induced conformational changes of NRs. Here, we describe a generally applicable bioluminescence assay with a genetically encoded bioluminescent indicator to determine androgenicity of ligands based on the intramolecular association of the ligand-binding domain of androgen receptor (AR LBD) with the "FQNLF" motif in the N-terminal domain of AR (AR NTD). FLuc was dissected into N-terminal (1–415 AA) and C-terminal (416–550 AA) fragments. The AR LBD and FQNLF motif of AR NTD were sandwiched between the dissected fragments of FLuc to construct a single-molecule-format bioluminescent probe. Androgens induce the association of AR LBD with the FQNLF motif in the NTD, and the subsequent complementation of N- and C-terminal fragments of FLuc partially restores the activities of FLuc. A 10^{-5} M solution of DHT induced a quick increase in the luminescence intensities from cervical carcinoma–derived HeLa cells carrying the genetic indicator, which reached a plateau in 9 min, whereas DHT withdrawal from the cells by a medium change decreased the luminescence more slowly (i.e., 2 h elapsed until luminescence returned to the background level) (61). The present luminescent indicator was found to exhibit high agonist selectivity and reproducible recovery of the luminescence to a repeated androgen addition and withdrawal. This is the first contribution that cellular signaling steps can be imaged with bioluminescence using a single-molecule-format bioluminescence probe (Simbi), in which all the components required for signal sensing and visualization are integrated. Simbi is applicable to developing biotherapeutic agents effective to the AR signaling, and for screening adverse chemicals that possibly influence the signal transduction of AR (17, 24, 26, 62–64).

Firefly luciferase connected with a substrate sequence for caspase-3 (DEVD) is cyclized by a DnaE intein. When the cyclic luciferase is expressed in living cells, its activity is greatly decreased because of a steric effect. Activated caspase-3 cleaves the substrate sequence in the cyclic luciferase and the luciferase activity is restored. Quantitative sensing of time-dependent caspase-3 activity in living cells and in mice upon the application of extracellular stimuli has been demonstrated (**Figure 8**) (65–71).

6. NUCLEAR RECEPTOR–COREGULATOR INTERACTIONS

A sensitive fluorescent indicator was designed to visualize, in real time, the activities of the AR ligands in the physiological environment of single living cells (17). An androgen promotes interaction between the androgen receptor ligand binding domain (AR LBD) and coactivator protein. This results in an increase in FRET from CFP to YFP. The indicator is capable of distinguishing ligands of different potencies for the AR. The present assay is intended to indicate not the binding affinity of a drug, but rather the efficacy of a drug as either an antagonist or partial agonist in vivo. The permeability of a drug into cells and the conformational changes induced in the AR all determine its efficacy, much more so than a simple binding assay. Progesterone, glucocorticoid, and peroxisome proliferator-activated receptors (PR, GR, and PPAR, respectively) also belong to the NR family and play important roles in mediating the

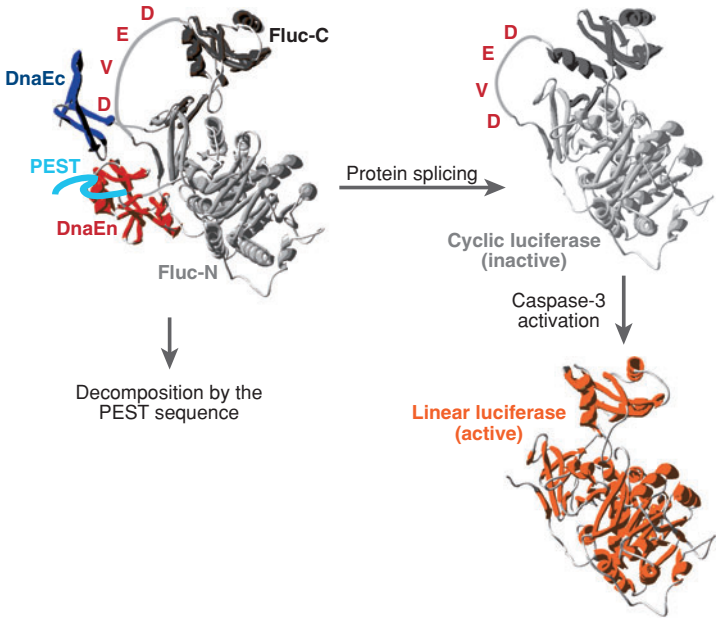


Figure 8

Strategy for the detection of caspase-3 activity. Principle for monitoring the activity of caspase-3 by using split firefly luciferase (Fluc). Abbreviation: PEST, prolinc, glutamic acid, serine, and threonine-rich.

actions of drugs for contraception (by PR), inflammation (by GR), and type-2 diabetes (by PPARγ). Using the present strategy, indicators for PR, GR, and PPARγ can be developed for screening and characterization of their ligands. The indicators would be helpful in the development of NR-based pharmaceutical drugs for the treatment of different diseases (**Figure 9**).

Selective nuclear receptor modulators (SNRMs), which are used clinically for the treatment of NR-related diseases, display mixed agonistic/antagonistic activity in a tissue-selective manner depending on the cellular concentrations of coregulator proteins, (e.g., coactivators and corepressors). The molecular details of the SNRM function provided us with an idea for a rational method for the high-throughput screening of SNRMs in real time in intact living cells. We have developed genetically encoded fluorescent indicators based on the principle of ligand-induced co-activator and/or corepressor recruitment to NR ligand-binding domain in single living cells. We demonstrated that an SNRM induces a distinct conformational change in the NR LBD, which differ from that induced by a full agonist or antagonist, favorable for the recruitment of a coactivator or corepressor protein to the NR. The molecular details of an SNRM-binding NR and the subsequently induced conformational changes are important to the understanding of SNRM action in the living body. Our fluorescent indicators are capable of distinguishing among agonists, antagonists, and SNRMs, and can therefore serve as versatile molecular sensors that predict

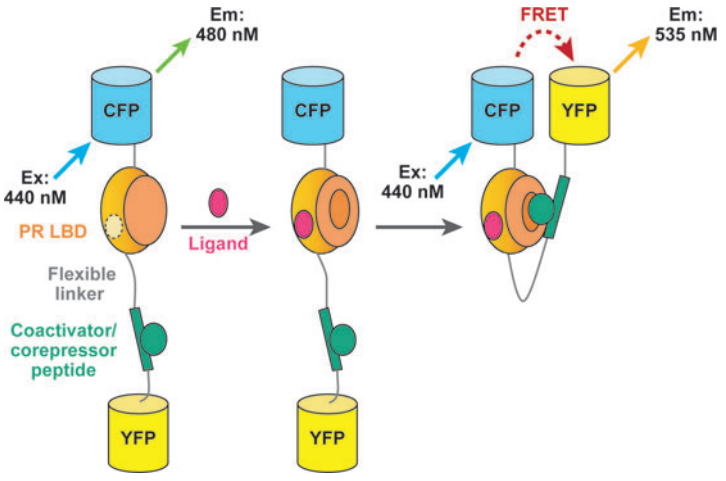


Figure 9

Fluorescent indicator for the ligand-induced coactivator/corepressor recruitment to the progesterone ligand binding domain (PR LBD) in living cells. Principle of the coactivator/corepressor-based ligand-induced/fluorescent indicator based on intramolecular fluorescence resonance energy transfer (FRET) to visualize the ligand-dependent interaction between the PR LBD and the steroid receptor coactivator 1 peptide/silencing mediator for retinoid and thyroid hormone receptor. Upon ligand binding, the PR LBD and coactivator/corepressor interact with each other. Consequently, yellow fluorescent protein (YFP) is oriented in close proximity to cyan fluorescent protein (CFP); this results in an increase in the FRET response. The magnitude of the FRET increase strongly depends on the relative orientation and distance between the donor (CFP) and acceptor (YFP) fluorophore.

the pharmacological character ligands, which is important for accurate treatment of disease (9, 72–75).

One member of the NR superfamily, PPAR (described above) plays an important role in modulation of insulin sensitivity in type 2 diabetes. Ligand-dependent protein-protein interactions between NRs and NR coactivators are critical in regulation of transcription. To visualize the ligand-induced coactivator recruitment to PPAR in live cells, we developed a genetically encoded fluorescent indicator in which PPAR ligand binding domain (PPAR LBD) was connected to a steroid receptor coactivator peptide that contains LXXLL motif (where L = leucine and X = any amino acid) through a flexible linker. This fusion protein was inserted between CFP and YFP, the donor and acceptor fluorophores, respectively. Monitoring real-time ligand-induced conformational change in the PPAR LBD to interact with the coactivator allowed screening of natural and synthetic ligands (drugs for the treatment of type 2 diabetes) in single living cells using intramolecular FRET microscopy. The high sensitivity of the present indicator made it possible to distinguish between strong and weak affinity ligands for PPAR in a dose-dependent fashion immediately after adding a ligand to live cells. The indicator can discriminate agonist from antagonist compounds efficiently within a few minutes. The present system may be promising for the development of PPAR-targeted drugs for type 2 diabetes and inflammation.

7. NUCLEOCYTOPLASMIC TRAFFICKING OF FUNCTIONAL PROTEINS

Nucleocytoplasmic trafficking of functional proteins plays a key role in regulating gene expressions in response to extracellular signals. A genetically encoded bioluminescent indicator was developed for monitoring the nuclear trafficking of target proteins in vitro and in vivo (24). The principle is based on reconstitution of split fragments of *Renilla reniformis* (Rluc) by protein splicing with a DnaE intein. A target cytosolic protein fused to the amino-terminal half of Rluc is expressed in mammalian cells. If the protein translocates into the nucleus, the Rluc moiety meets the C-terminal half of Rluc, which is localized in the nucleus with a fused NLS, and full-length Rluc is reconstituted by protein splicing. The bioluminescence is thereby emitted with coelenterazine as the substrate. The principle of the approach is an extension of the method developed earlier for identifying mitochondrial proteins (22) (**Figure 10**).

The method of cell-based screening with the genetically encoded indicator provided a quantitative measure of the extent of nuclear translocation of AR upon stimulation with various chemicals. Currently, high-throughput screening tools for protein translocation into the nucleus have mostly depended upon the GFP- (or its variant) tagged approach in combination with the fluorescence microscopy and computer-driven imaging system. The system offers only semiquantitative information, as it is difficult to accurately distinguish the fluorescence of GFP-tagged proteins localized only in the nucleus from that left in the cytosol. In addition, the precision of the observed fluorescence intensities from the nucleus obtained with the statistical analysis is not high because the number of cells examined under a

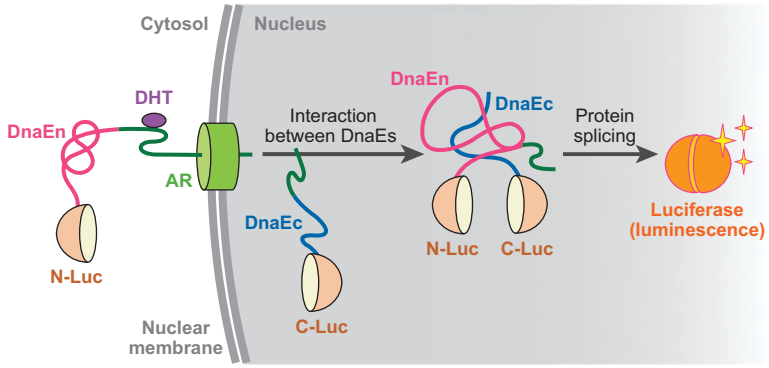


Figure 10

When androgen receptor (AR) is bound to 5α-dihydrotestosterone (DHT), it translocates into the nucleus and brings the N- and C-terminal halves of DnaEs close enough to fold correctly, thereby initiating protein splicing to link the concomitant Rluc halves with a peptide bond. The C-terminal half of split Rluc was located beforehand in the nucleus by a fused nuclear localization signal. The cells containing this reconstituted Rluc allow monitoring of nuclear translocation of AR with its luminescence using coelenterazine as the substrate.

fluorescence microscope is limited. However, the present method enabled determination of the subcellular localization of AR by the luminescence signals generated only when the AR localized in the nucleus. AR remaining in the cytosol did not induce reconstitution of split Rluc and therefore no background luminescence was observed (24).

Polychlorinated biphenyl (PCB) and procymidone have been suspected of having neurotoxic and antiandrogenic effects, respectively, and they possibly adversely influence hormonal activities in living animals' brains. We demonstrated the usefulness of the split rLuc reporter for monitoring AR translocation into the nucleus in living mice by implanting COS-7 cells in the mouse brain at a depth of 3 mm and measuring emitted bioluminescence with a cooled CCD camera; we thereby investigated the distribution of these chemicals in the brains of living mice. As expected, 2 h after intraperitoneally injecting PCB or procymidone, both chemicals were found to completely inhibit the DHT-stimulated translocation of AR, where coelenterazine was injected intracerebrally (24).

Similar genetically encoded bioluminescent probes were developed for illuminating protein nuclear transport induced by phosphorylation or proteolysis (25). A genetically encoded stress indicator was also reported as noninvasively imaging endogenous corticosterone in living mice (26) (**Figure 11**).

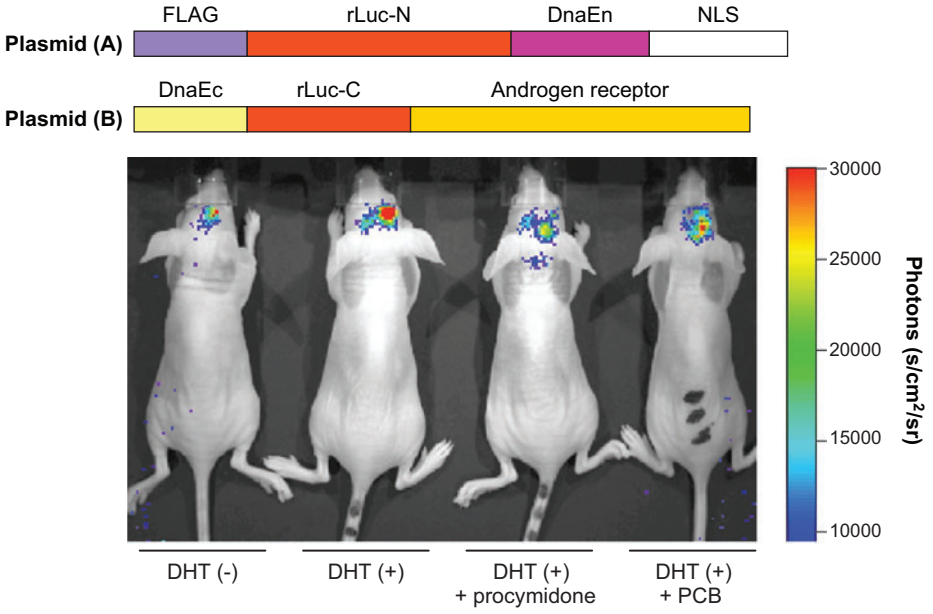


Figure 11

Effects of inhibitors on androgen receptor (AR) translocation into the nucleus in the mouse brain. Polychlorinated biphenyl (PCB) and procymidone were found to have an ability to pass through the blood-brain barrier, to reach the brain, and to inhibit the AR signal transduction in the organ.

8. PROTEIN LOCALIZATIONS

Smac/DIABLO is an intermembrane space– (IMS-)localized protein that is conserved in mammals (76, 77). The Smac/DIABLO protein derived from *Mus musculus* is synthesized as a precursor molecule of 237 amino acids; the N-terminal 53 residue serves as the mitochondrial targeting sequence, which is removed by the inner membrane peptidase complex after import (78). Thus, the mature Smac/DIABLO protein has 184 amino acids, and the four residues of the N terminus are Ala-Val-Pro-Ile. These four N-terminal residues play an indispensable role in Smac/DIABLO function: They promote apoptosis by eliminating the inhibitory effect of the inhibitor of apoptosis protein (IAP) through physical interaction (79–81). A point mutation in the four residues leads to a loss of interaction with IAP and a concomitant loss of the Smac/DIABLO function. The structural and functional aspects of Smac/DIABLO have been extensively investigated, but how the protein targets into the mitochondrial IMS remains unknown. To identify the amino acids or domains of Smac/DIABLO that are important for targeting into the IMS, we have developed a high-throughput screening system that enables discrimination between the proteins in the IMS and

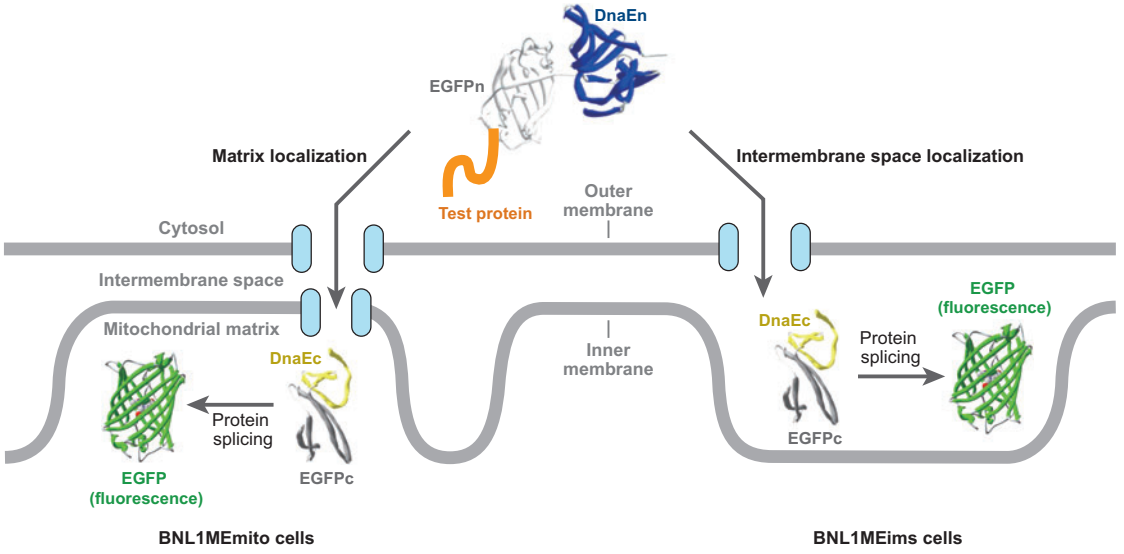


Figure 12

Enhanced green fluorescent protein (EGFP) reconstitution by protein splicing in submitochondrial compartments. When a test protein is localized in the mitochondrial matrix (*left*) or in the intermembrane space (IMS) (*right*), N- and C-terminal DnaE are brought close together, and EGFP is formed by protein splicing in the respective compartments. BNL1MEmito cells permanently express a fusion composed of C-terminal fragments of DnaE and EGFP in the mitochondrial matrix (*left*), and BNL1MEims cells express the same fusion in the IMS (*right*). The colored three-dimensional structures represent DnaEn and DnaEc, and the gray structures represent N- and C-terminal fragments of EGFP, respectively. The attached strand represents a test protein.

those in the mitochondrial matrix or cytosol. Using this system, we identified from Smac/DIABLO mutant libraries the amino acids necessary for the localization of the IMS. Further, we showed that amino acid residues 54–57 (Ala-Val-Pro-Ile), which are crucial for the apoptotic function, are also important for the IMS localization. We found that N-terminal amino acid residues 10–57 (RSVCSLFRYRQRFPVLAN-SKKRCFSELIKPWHKTVLTGFGMTLCAVPI) are the minimal sequence that functions as the IMS-targeting signal. We demonstrated that this IMS-targeting signal is able to deliver different probe proteins and intrabodies into the IMS (**Figure 12**).

We developed genetically encoded RNA probes for characterizing localization and dynamics of mitochondrial RNA (mtRNA) in single living cells. The probes consist of two RNA-binding domains of PUMILIO1, each connected with split fragments of a fluorescent protein capable of reconstituting upon binding to a target RNA. We

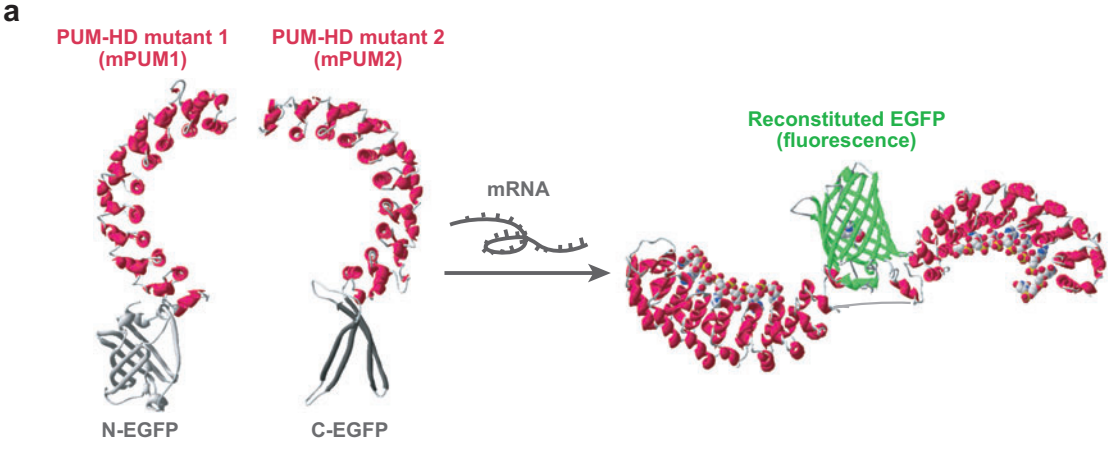


Figure 13

Detection of a target mRNA based on complementation of split enhanced green fluorescent protein (EGFP) fragments. (*a*) Schematic of the basic strategy for detecting target mRNAs. Two RNA-binding domains of pumilio (PUM)-HD are engineered to recognize specific sequences on a target mRNA (mPUM1-RNA and mPUM2-RNA). In the presence of the target mRNA, mPUM1 and mPUM2 bind to their target sequences, bringing together the N- and C-terminal fragments of EGFP. This results in functional reconstitution of the fluorescent protein. (*b*) Sequences of mitochondrial RNA (mtRNA) that are recognized by PUM-HD mutants.

designed the probes to specifically recognize a 16-base sequence of mtRNA-encoding nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 6 (ND6) and to be targeted into the mitochondrial matrix, which allowed real-time imaging of ND6 mtRNA localization in living cells. We showed that ND6 mtRNA is localized within mitochondria and is concentrated particularly on mitochondrial DNA. Movement of the ND6 mtRNA is restricted, but oxidative stress induces the mtRNA to disperse in the mitochondria and gradually decompose. These probes provide a means of studying spatial and temporal mtRNA dynamics in intracellular compartments in living mammalian cells (82) (**Figure 13**).

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED

1. Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–50
2. Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirin Y, et al. 1998. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem.* 70:2446–53
3. Komatsu H, Iwasawa N, Citterio D, Suzuki Y, Kubota T, et al. 2004. Design and synthesis of highly sensitive and selective fluorescein-derived magnesium fluorescent probes and application to intracellular 3D Mg^{2+} imaging. *J. Am. Chem. Soc.* 126:16353–60
4. Hirano T, Kikuchi K, Urano Y, Higuchi T, Nagano T. 2000. Novel zinc fluorescent probes excitable with visible light for biological applications. *Angew. Chem. Int. Ed.* 39:1052–54
5. Chalfie M, Kain S, eds. 1998. *Green Fluorescent Protein: Properties, Applications, and Protocols*. New York: Wiley-Liss
6. Zacharias DA, Baird GS, Tsien RY. 2000. Recent advances in technology for measuring and manipulating call signals. *Curr. Opin. Neurobiol.* 10:416–21
7. Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, et al. 1997. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* 388:882–87
8. Zaccolo M, Giorgi FD, Cho CY, Feng L, Knapp T, et al. 2000. A genetically encoded, fluorescent indicator for cyclic AMP in living cells. *Naure Cell Biol.* 2:25–29

9. Sato M, Hida N, Umezawa Y. 2005. Imaging the nanomolar range of nitric oxide with an amplifier-coupled fluorescent indicator in living cells. *Proc. Natl. Acad. Sci. USA* 102:14515–20
10. Hirose K, Kadowaki S, Tanabe M, Takeshima H, Iino M. 1999. Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca^{2+} mobilization patterns. *Science* 284:1527–30
11. Sato M, Ueda Y, Shibuya M, Umezawa Y. 2005. Locating inositol 1,4,5-trisphosphate in the nucleus and neuronal dendrites with genetically encoded fluorescent indicators. *Anal. Chem.* 77:4751–58
12. Sato M, Hida N, Ozawa T, Umezawa Y. 2000. Fluorescent indicators for cyclic GMP based on cyclic GMP-dependent protein kinase Iα and green fluorescent proteins. *Anal. Chem.* 72:5918–24
13. Sato M, Ueda Y, Takagi T, Umezawa Y. 2003. Production of PtdInsP3 at endomembranes is triggered by receptor endocytosis. *Nat. Cell Biol.* 5:1016–22
14. Sato M, Ozawa T, Inukai K, Asano T, Umezawa Y. 2002. Fluorescent indicators for imaging protein phosphorylation in single living cells. *Nat. Biotechnol.* 20:287–94
15. Sasaki K, Sato M, Umezawa Y. 2003. Fluorescent indicators for Akt/protein kinase B and dynamics of Akt activity visualized in living cells. *J. Biol. Chem.* 247:30945–51
16. Sato M, Umezawa Y. 2004. Imaging protein phosphorylation by fluorescence in single living cells. *Methods* 32:451–55
17. Awais M, Sato M, Lee X, Umezawa Y. 2006. A fluorescent indicator to visualize activities of the androgen receptor ligands in single living cells. *Angew. Chem. Int. Ed.* 45:2707–12
18. Ozawa T, Nogami S, Sato M, Ohya Y, Umezawa Y. 2000. A fluorescent indicator for detecting protein-protein interactions in vivo based on protein splicing. *Anal. Chem.* 72:5151–57
19. Ozawa T, Kaihara A, Sato M, Tachihara K, Umezawa Y. 2001. Split luciferase as an optical probe for detecting protein-protein interactions in mammalian cells based on protein splicing. *Anal. Chem.* 73:2516–21
20. Kaihara A, Kawai Y, Sato M, Ozawa T, Umezawa Y. 2003. Locating a protein-protein interaction in living cells via split *Renilla* luciferase complementation. *Anal. Chem.* 75:4176–81
21. Kanno A, Ozawa T, Umezawa Y. 2006. Intein-mediated reporter gene assay for detecting protein-protein interactions in living mammalian cells. *Anal. Chem.* 78:556–60
22. Ozawa T, Sako Y, Sato M, Kitamura T, Umezawa Y. 2003. Intein-mediated reporter gene assay for detecting protein-protein interactions in living mammalian cells. *Nat. Biotechnol.* 21:287–93
23. Ozawa T, Nishitani K, Sako Y, Umezawa Y. 2005. A high-throughput screening of genes that encode proteins transported into the endoplasmic reticulum in mammalian cells. *Nucleic Acids Res.* 33:e34
24. Kim SB, Ozawa T, Watanabe S, Umezawa Y. 2004. High-throughput sensing and noninvasive imaging of protein nuclear transport by using reconstitution of split *Renilla* luciferase. *Proc. Natl. Acad. Sci. USA* 101:11542–47

25. Kim SB, Takao R, Ozawa T, Umezawa Y. 2005. Quantitative determination of protein nuclear transport induced by phosphorylation or by proteolysis. *Anal. Chem.* 77:6928–34
26. Kim SB, Ozawa T, Umezawa Y. 2005. Genetically encoded stress indicator for noninvasively imaging endogenous corticosterone in living mice. *Anal. Chem.* 77:6588–93
27. Sato M, Nakajima T, Goto M, Umezawa Y. 2006. A cell-based indicator visualizes picomolar dynamics of nitric oxide release from living cells. *Anal. Chem.* 78:8175–82
28. Losel R, Wehling M. 2003. Nongenomic actions of steroid hormones. *Nat. Rev. Mol. Cell Biol.* 4:46–56
29. Marquez DC, Pietras RJ. 2001. Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. *Oncogene* 20:5420–30
30. Kousteni S, Chen JR, Bellido T, Han L, Ali AA, et al. 2002. Reversal of bone loss in mice by nongenotropic signaling of sex steroids. *Science* 298:843–46
31. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, et al. 2000. Steroid-induced androgen receptor-oestradiol receptor β-Src complex triggers prostate cancer cell proliferation. *EMBO J.* 19:5406–17
32. Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, et al. 2001. PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J.* 20:6050–59
33. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, et al. 1996. Tyrosine kinase/p21(ras)/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J.* 15:1292–300
34. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, et al. 2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 104:719–30
35. Simons K, Toomre D. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1:31–39
36. Cary LA, Cooper JA. 2000. Molecular switches in lipid rafts. *Nature* 404:945–47
37. Sargiacomo M, Sudol M, Tang Z, Lisanti MP. 1993. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* 122:789–807
38. Liang X, Nazarian A, Erdjument-Bromage H, Bornmann W, Tempst P, Resh MD. 2001. Heterogeneous fatty acylation of Src family kinases with polyunsaturated fatty acids regulates raft localization and signal transduction. *J. Biol. Chem.* 276:30987–94
39. Hitosugi T, Sasaki K, Sato M, Umezawa Y. 2007. Epidermal growth factor directs sex-specific steroid signaling through Src activation. *J. Biol. Chem.* 282:10697–706
40. Fields S, Song OK. 1989. A novel genetic system to detect protein protein interactions. *Nature* 340:245–46
41. Chien CT, Bartel PL, Sternglanz R, Fields S. 1991. The 2-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA* 88:9578–82

42. Dang CV, Barrett J, Villagarcia M, Resar LMS, Kato GJ, Fearon ER. 1991. Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. *Mol. Cell. Biol.* 11:954–62
43. Fearon ER, Finkel T, Gillison ML, Kennedy SP, Casella JF, et al. 1992. Karyoplasmic interaction selection strategy: a general strategy to detect protein-protein interactions in mammalian cells. *Proc. Natl. Acad. Sci. USA* 89:7958–62
44. Flores A, Briand JF, Gadal O, Andrau JC, Rubbi L, et al. 1999. A protein-protein interaction map of yeast RNA polymerase III. *Proc. Natl. Acad. Sci. USA* 96:7815–20
45. Ito T, Tashiro K, Muta S, Ozawa R, Chiba T, et al. 2000. Toward a protein-protein interaction map of the budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *Proc. Natl. Acad. Sci. USA* 97:1143–47
46. Walhout AJM, Sordella R, Lu XW, Hartley JL, Temple GF, et al. 2000. Protein interaction mapping in *C. elegans* using proteins involved in vulval development. *Science* 287:116–22
47. Johnsson N, Varshavsky A. 1994. Split ubiquitin as a sensor of protein interactions in-vivo. *Proc. Natl. Acad. Sci. USA* 91:10340–44
48. Stagljar I, Korostensky C, Johnsson N, te Heesen S. 1998. A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. *Proc. Natl. Acad. Sci. USA* 95:5187–92
49. Dunnwald M, Varshavsky A, Johnsson N. 1999. Detection of transient in vivo interactions between substrate and transporter during protein translocation into the endoplasmic reticulum. *Mol. Biol. Cell* 10:329–44
50. Luker KE, Smith MCP, Luker GD, Gammon ST, Piwnica-Worms H, et al. 2004. Kinetics of regulated protein-protein interactions revealed with firefly luciferase complementation imaging in cells and living animals. *Proc. Natl. Acad. Sci. USA* 101:12288–93
51. Paulmurugan R, Gambhir SS. 2003. Monitoring protein-protein interactions using split synthetic *Renilla* luciferase protein-fragment-assisted complementation. *Anal. Chem.* 75:1584–89
52. Ozawa T, Takeuchi M, Kaihara A, Sato M, Umezawa Y. 2001. Protein splicing–based reconstitution of split green fluorescent protein for monitoring protein-protein interactions in bacteria: improved sensitivity and reduced screening time. *Anal. Chem.* 73:5866–74
53. Hirata R, Ohsumi Y, Anraku Y. 1989. Functional molecular masses of vacuolar membrane H^+-ATPase from *Saccharomyces cerevisiae* as studied by radiation inactivation analysis. *FEBS Lett.* 244:397–401
54. Kane PM, Yamashiro CT, Wolczyk DF, Neff N, Goebl M, et al. 1990. Protein splicing converts the yeast TFP1 gene-product to the 69-kD subunit of the vacuolar $H^{(+)}$-adenosine triphosphatase. *Science* 250:651–57
55. Noren CJ, Wang JM, Perler FB. 2000. Dissecting the chemistry of protein splicing and its applications. *Angew. Chem. Int. Ed.* 39:450–66
56. Paulus H. 2000. Protein splicing and related forms of protein autoprocessing. *Annu. Rev. Biochem.* 69:447–96

57. Cooper AA, Chen YJ, Lindorfer MA, Stevens TH. 1993. Protein splicing of the yeast TFP1 intervening protein-sequence: a model for self-excision. *EMBO J.* 12:2575–83
58. Chong SR, Xu MQ. 1997. Protein splicing of the *Saccharomyces cerevisiae* VMA intein without the endonuclease motifs. *J. Biol. Chem.* 272:15587–90
59. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, et al. 2000. Steroid-induced androgen receptor–oestradiol receptor-Src complex triggers prostate cancer cell proliferation. *EMBO J.* 19:5406–17
60. Luker KE, Smith MC, Luker GD, Gammon ST, Piwnica-Worms H, Piwnica-Worms D. 2004. Kinetics of regulated protein-protein interactions revealed with firefly luciferase complementation imaging in cells and living animals. *Proc. Natl. Acad. Sci. USA* 101:12288–93
61. Kim SB, Otani Y, Umezawa Y, Tao H. 2007. Bioluminescent indicator for determining protein/protein interactions using intramolecular complementation of split click beetle luciferase. *Anal Chem.* 79:4820–26
62. Schaufele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, et al. 2005. The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. *Proc. Natl. Acad. Sci. USA* 102:9802–7
63. Langley E, Zhou ZX, Wilson EM. 1995. Evidence for an antiparallel orientation of the ligand-activated human androgen receptor dimmer. *J. Biol. Chem.* 270:29983–90
64. He B, Kemppainen JA, Wilson EM. 2000. FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. *J. Biol. Chem.* 275:22986–94
65. Reits E, Griekspoor A, Neijssen J, Groothuis T, Jalink K, et al. 2003. Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* 18:97–108
66. Nagai T, Miyawaki A. 2004. A high-throughput method for development of FRET-based indicators for proteolysis. *Biochem. Biophys. Res. Commun.* 319:72–77
67. Takemoto K, Nagai T, Miyawaki A, Miura M. 2003. Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects. *J. Cell Biol.* 160:235–43
68. Xu X, Gerard ALV, Huang BCB, Anderson DC, Payan DG, et al. 1998. Detection of programmed cell death using fluorescence energy transfer. *Nucleic Acids Res.* 26:2034–35
69. Mahajan NP, Harrison-Shostak DC, Michaux J, Herman B. 1999. Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis. *Chem. Biol.* 6:401–9
70. Weissleder R, Ntziachristos V. 2003. Shedding light onto live molecular targets. *Nat. Med.* 9:123–28
71. Laxman B, Hall DE, Bhojani MS, Hamstra DA, Chenevert TL, et al. 2002. Noninvasive real-time imaging of apoptosis. *Proc. Natl. Acad. Sci. USA* 99:16551–55

72. Xu HE, Stanley TB, Montana VG, Lambert MH, Shearer BG, et al. 2002. Structural basis for antagonist-mediated recruitment of nuclear corepressors by PPAR. *Nature* 415:813–17
73. Williams SP, Sigler PB. 1998. Transcriptional activation independent of TFIIH kinase and the RNA polymerase II mediator in vivo. *Nature* 393:392–96
74. Heery DM, Kalkhoven E, Hoare S, Parker MG. 1997. A signature motif in transcriptional coactivators mediates binding to nuclear receptor. *Nature* 387:733–36
75. Onate SAO, Tsai SY, Tsai MU, O'Malley BW. 1995. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270:1354–57
76. Du C, Fang M, Li Y, Li L, Wang X. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102:33–42
77. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, et al. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102:43–53
78. Burri L, Strahm Y, Hawkins CJ, Gentle IE, Puryer MA, et al. 2005. Mature DIABLO/Smac is produced by the IMP protease complex on the mitochondrial inner membrane. *Mol. Biol. Cell* 16:2926–33
79. Liu Z, Sun C, Olejniczak ET, Meadows RP, Betz SF, et al. 2000. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* 408:1004–8
80. Chai J, Du C, Wu JW, Kyin S, Wang X, Shi Y. 2000. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 406:855–62
81. Wu G, Chai J, Suber TL, Wu JW, Du C, et al. 2000. Structural basis of IAP recognition by Smac/DIABLO. *Nature* 408:1008–12
82. Ozawa T, Natori Y, Sato M, Umezawa Y. 2007. Imaging dynamics of endogenous mitochondrial RNA in single living cells. *Nature Methods* 4:413–19