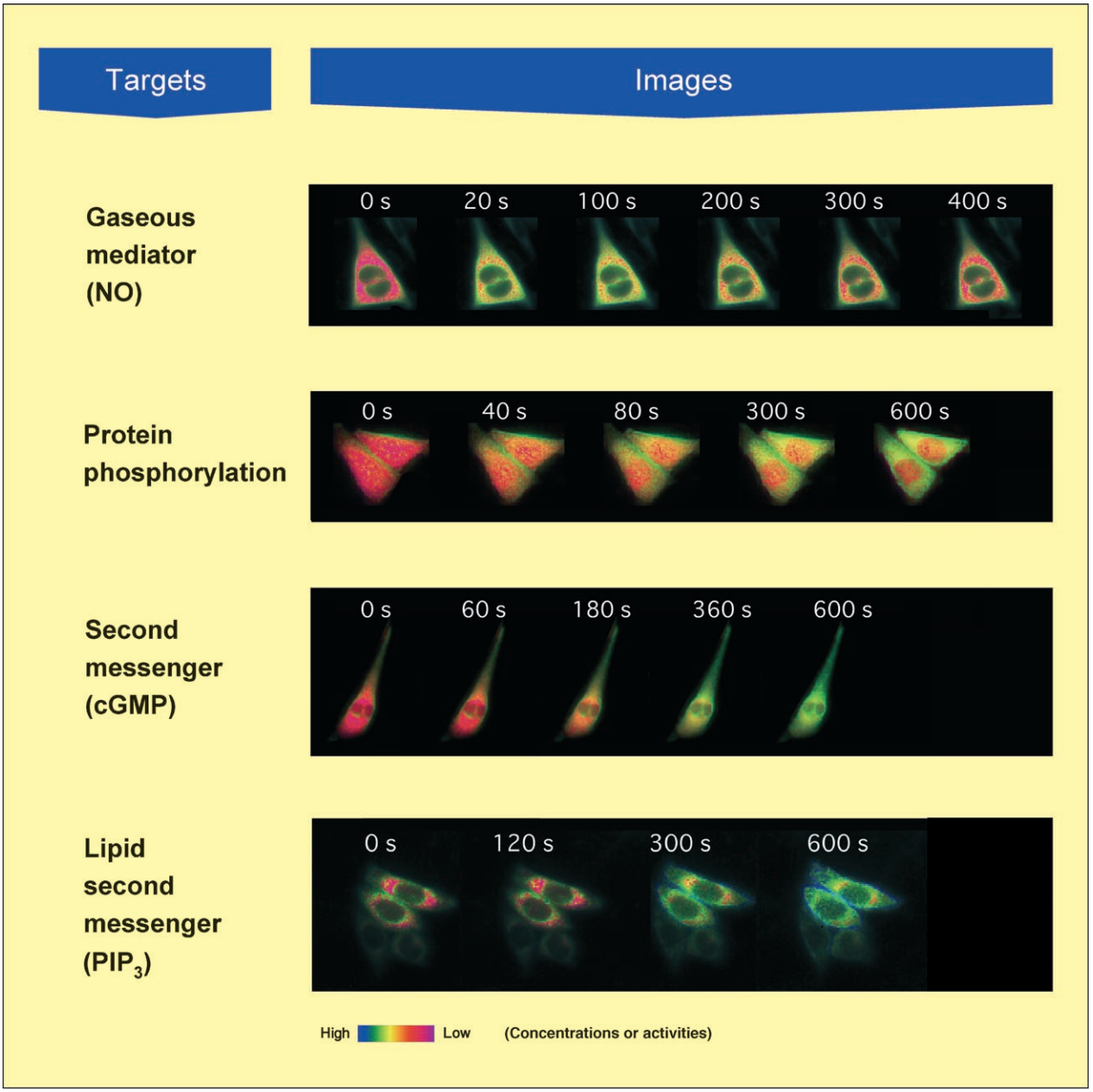
DOI: 10.1002/asia.200600063

Illuminating Molecular Processes in Living Cells

Yoshio Umezawa*[a]



Abstract: Lately, scientists have explored approaches to developing fluorescent and/or bioluminescent indicators to pinpoint cellular processes in single living cells. These analytical methods have become a key technology for visualizing and detecting what was otherwise unseen in live cells. The target signaling included second messengers, protein phosphorylations, protein–protein interactions, and protein localizations.

Keywords: cellular signaling · FRET (fluorescence resonant energy transfer) · imaging agents · optical probes · phosphorylation

1. Introduction

Binding assays are commonly used for the analysis of bioactive substances. Conventional binding assays can neither discriminate agonists from antagonists nor give sufficient information on their physiological activities. Physical methods such as NMR spectroscopy and MS cannot provide such information either. Bioassays with intact biological tissue or whole bodies have a unique position in analysis, because they can target bioactive substances. However, bioassays cannot give information at the molecular level because of their inherent "black-box" approach.

During the past 50 years, molecular biology has developed mostly by taking advantage of physical and chemical methods of analysis, and thereby contributed to elucidate the molecular chemistry behind cellular mechanisms. If analytical methods for bioactive substances are based not only on binding to receptors but also on following the known molecular-level processes of signal transduction in the respective signaling pathways, reconstructed in vitro or taken in part in vivo, they will give the physiologically relevant selectivities of the analytes in terms of cellular mechanisms at the molecular level. This is of prime importance for screening and targeting pharmaceutically, toxicologically, and environmentally relevant bioactive substances.

[a] Prof. Dr. Y. Umezawa
Department of Chemistry
School of Science
The University of Tokyo
Hongo, Bunkyo-Ku, Tokyo 113-0033 (Japan)
Fax: (+81) 3-5841-8349
E-mail: umezawa@chem.s.u-tokyo.ac.jp

2. Probing Cellular Signaling Pathways in Living Cells

For nondestructive analysis of chemical processes in living cells, organic fluorescent probe molecules have been developed for ions and small molecules such as Ca^{2+},[1] NO,[2] Mg^{2+},[3] and Zn^{2+}.[4] Furthermore, green fluorescent protein (GFP) and its analogous proteins have been used to probe proteins as to their structural and locational changes, upon genetically labeling them to proteins of interest.[5]

Many intracellular chemical processes and cellular-signaling processes are still studied essentially by relying on destructive analysis involving the disruption of hundreds of thousands of cells followed by separation, purification, and detection of intracellular components. Therefore, methods for direct nondestructive analysis of cellular-signaling steps in live cells are highly required.

Intercellular-signaling substances include neurotransmitters, cytokines, and hormones functioning in nervous, 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 signal.

The intracellular signal can be monitored in vivo in living cells[6] by genetically encoded intracellular fluorescent and bioluminescent probes or indicators (Figure 1). Scientists have reported a number of such probes for visualizing cellular signaling. The probes include second messengers such as Ca^{2+},[7] cAMP,[8] nitric oxide (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] Only several representative examples are illustrated below.

These probes are of general use not only for fundamental biological studies, but also for assay and screening of possible pharmaceutical or toxic chemicals that inhibit or facilitate cellular-signaling pathways.

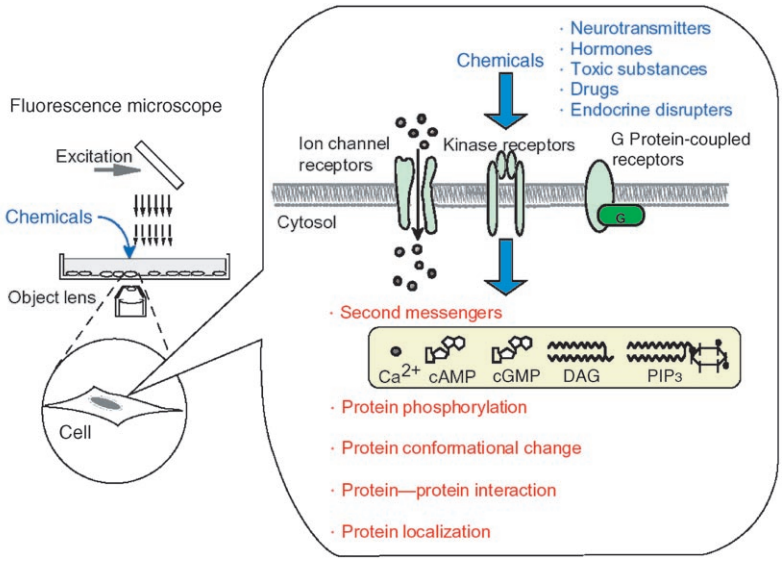


Figure 1. Imaging cellular signaling in single living cells. Genetically encoded fluorescent or bioluminescent probes for each signaling process are constructed and directly expressed in target cells. Upon stimulation of the cells with potential pharmaceuticals or possible toxic chemicals, the increase or decrease in fluorescent or bioluminescent signals are observed, which is a measure of the extent of promotion or inhibition of cellular signaling.

2.1 Nitric Oxide

Nitric oxide (NO) is a small uncharged free radi'cal that is involved in diverse physiological and pathophysiological mechanisms. NO is generated by three isoforms of NO synthase (NOS): endothelial, neuronal, and inducible. When generated in vascular endothelial cells, NO plays a key role in vascular-tone regulation in particular. An amplifier-coupled fluorescent indicator for NO was developed to visualize the physiological nanomolar dynamics of NO in living cells (detection limit of 0.1 nM) (Figure 2). Earlier, a cGMP fluorescent indicator, CGY, was developed, which in this case is combined with soluble guanylate cyclase (sGC) for the amplified detection of NO in living cells. This amplifier-coupled fluorescent indicator was named NOA-1 and binds with a single NO molecule to generate a large amount of cGMP in single living cells. This boosted amount of cGMP is detected in situ by the cGMP fluorescence resonant energy transfer (FRET) sensor built in NOA-1. NOA-1 unbound to cGMP does not emit FRET signals. Images of two cells expressed with the amplifier-coupled fluorescent indicator NOA-1 for nitric oxide are shown in Figure 3. The pseudocolor represents the cellular concentration of NO. The vascular endothelial cell stably generates 1 nM of the basal NO, compared to other cells such as CHO-K1. This genetically encoded highly sensitive 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 concentration of basal endothelial NO thus appears to be fundamental to vascular homeostasis.[9]

2.2 Phosphatidylinositol-3,4,5-trisphosphate

Phosphatidylinositol-3,4,5-trisphosphate (PIP_3) regulates diverse cellular functions, including cell proliferation and apoptosis, and plays a role 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 (Figure 4).[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 the artifacts described above. Furthermore, these indicators were fused with localization sequences to direct them to the plasma membrane or endomembranes, thus allowing localized analysis of PIP_3 concentrations. We used these fluorescent indicators to analyze the spatiotemporal regulation of PIP_3 production in single living cells. To examine PIP_3 dynamics, a pleckstrin homology domain (PHD) from GRP1 (general receptor for phosphoinositides-1) was used, which selective-

Abstract in Japanese:

最近科学者は，細胞情報過程をピンポイント可視化する技術を研究している．その結果，開発された蛍光／生物発光プローブは，生きた細胞中の“見えなかったことを見えるようにする”キーテクノロジーになっている．

Editorial Board Member

Yoshio Umezawa *is a Professor at the Univ. of Tokyo. His current research interests involve optical probes for visualizing molecular processes in living cells and intermolecular tunneling microscopy with STM molecular tips. His awards include the Japan Society for Analytical Chemistry Award (2000), the Spectroscopic Society of Japan Award (2005), and the Chemical Society of Japan Award (2006).*

"I wish Chemistry—An Asian Journal *all the best for its voyage towards becoming a renowned world-class journal."*

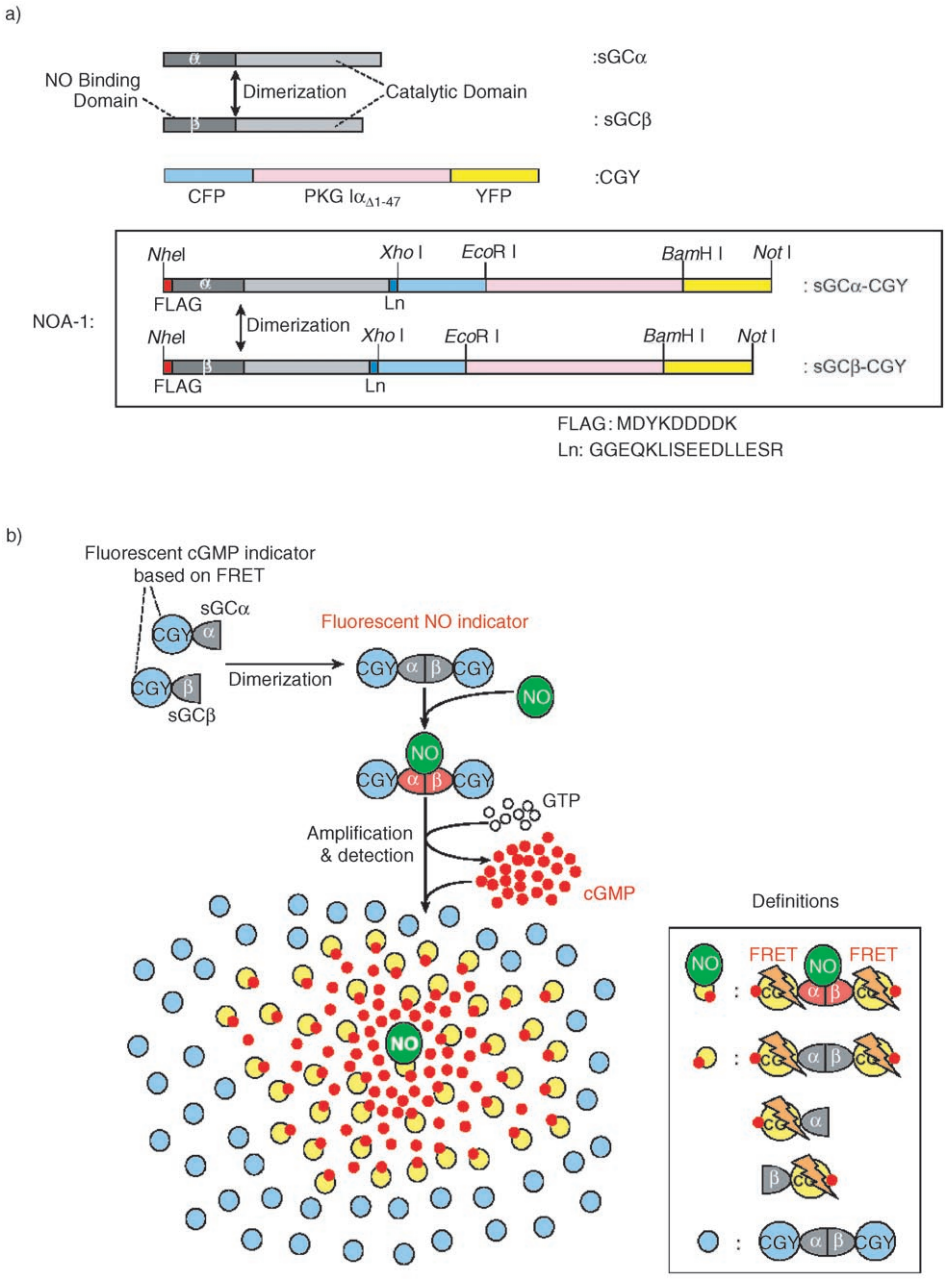


Figure 2. An amplifier-coupled fluorescent indicator for visualizing NO in single living cells. a) Schematic representations of the domain structures of sGCa, sGCb, CGY, sGCa–CGY and sGCb–CGY. The amino acid sequence of the FLAG tag and the linker (Ln) is shown at the bottom. The heterodimer of sGCa–CGY and sGCb–CGY was named NOA-1. b) Principle of the present NO indicator, NOA-1. sGCa–CGY and sGCb–CGY are spontaneously associated to form a matured heterodimer, that is, NOA-1. NOA-1 binds with NO and generates cGMP at the rate of 3000–6000 molecules min^{-1}. The generated cGMP binds to the CGY domain in NOA-1 make NOA-1 emit a FRET signal. Approximately 99.9% of cGMP molecules thus generated diffuse and bind to NO-free NOA-1. As a result, even a single NO molecule can trigger a large number of NOA-1 to emit FRET signals. Even if sGCa–CGY and sGCb–CGY exist as monomers, the monomers also emit FRET signals upon binding with generated cGMP.[9]

ly binds PIP_3, fused between cyan (CFP) and yellow (YFP) fluorescent protein variants, through rigid α-helical linkers consisting 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 (MLS) through the rigid α-helical linker. Thus, after phosphatidylinositol-3-OH kinase (PI(3)K) activation, the PHD 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 causes intramolecular FRET from CFP to YFP, thus allowing detection of PIP_3 dynamics at the membrane (Figure 4). We named this indicator "fllip" (fluorescent indicator for a lipid second messenger that can be tailor-made). The developed fllip allows the spatiotemporal examination of PIP_3 production in single living cells. After ligand stimulation, PIP_3 levels increased to a larger extent at the endomembranes (that is, the endoplasmic reticulum and the Golgi bodies) than at the plasma membrane (Figure 5). This increase was found to originate from in situ production at the endomembranes, a process stimulated directly by receptor tyrosine kinases produced by endocytosis from the plasma membrane to the endomembranes. The demonstration of PIP_3 production through receptor endocytosis addressed a long-standing question about how signaling pathways downstream of PIP_3 are activated at intracellular compartments remote from the plasma membrane.

3. Protein Phosphorylation

Protein phosphorylation by intracellular kinases plays one of the most pivotal roles in signaling pathways within cells. The kinase proteins catalyze transfer of a phosphate group from adenosine triphosphate (ATP) and phosphorylation of the hydroxy groups of serines, threonines, and/or tyrosines on the substrate proteins.

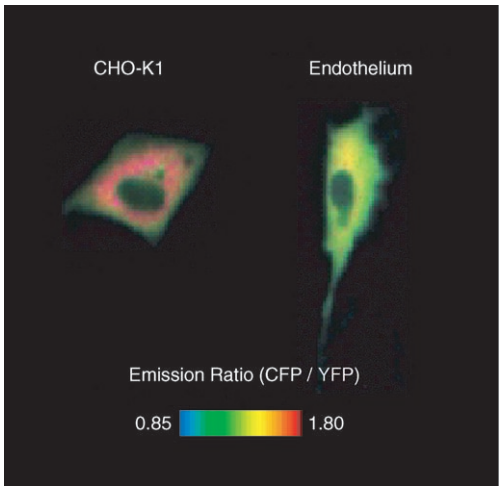


Figure 3. The pseudocolor represents the cellular concentration of NO. The vascular endothelial cell stably generates 1 nM of the basal NO, compared to other cells such as CHO-K1.

Upon this phosphorylation, the substrate proteins are subject to conformational changes caused by the negative charges of the phosphates which subsequently trigger their enzymatic activation and interaction with their respective target proteins. Electrophoresis, immunocytochemistry, and in vitro kinase assays have been used to investigate biological issues related to the kinase proteins. 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 of investigating kinase signaling, genetically encoded fluorescent indicators have been developed for visualizing protein phosphorylation in living cells.[14] The principle of the present method is shown in Figure 6. A substrate domain for a kinase protein of interest is fused to a phosphorylation recognition domain by means of a flexible linker sequence. The tandem fusion unit consisting of the substrate domain, linker sequence, and phosphorylation recognition domain is sandwiched between two fluorescent proteins of different colors, CFP and YFP, which serve as the donor and acceptor fluorophores for FRET. As a result of the phosphorylation of the substrate domain and subsequent binding of the phosphorylated substrate domain with the adjacent phosphorylation recognition domain, FRET is induced between the two fluorescent units which

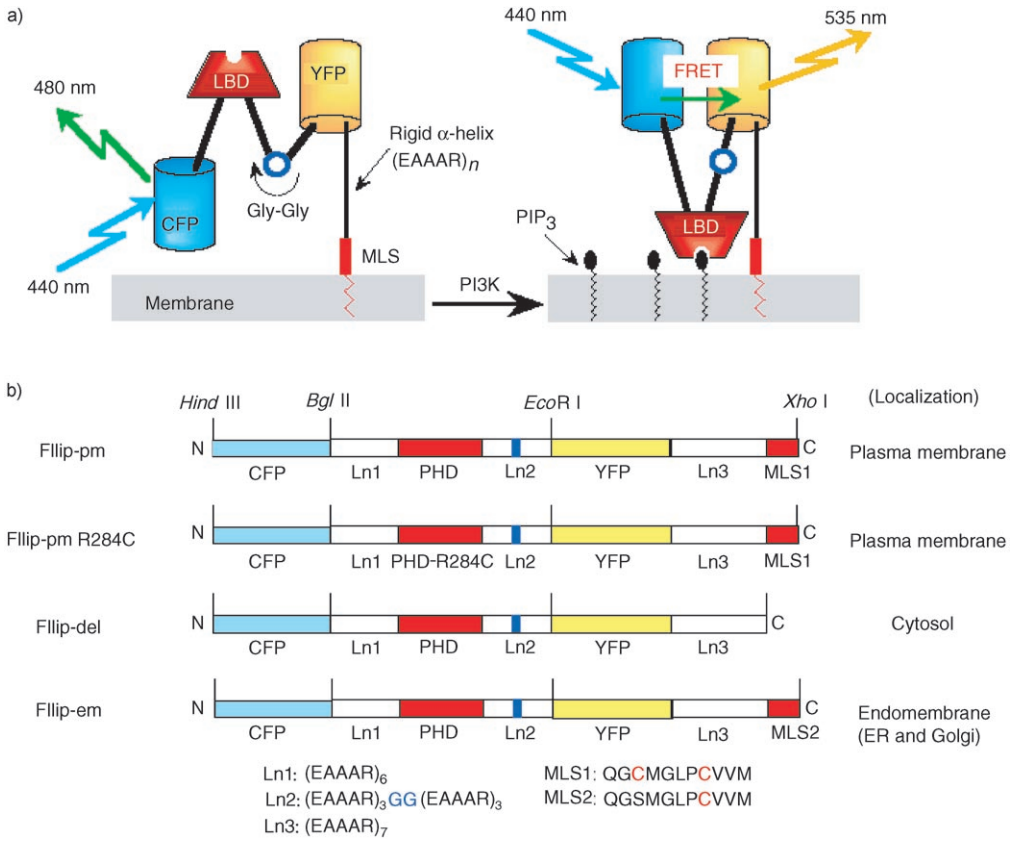


Figure 4. Fluorescent indicators for PIP_3 in single living cells. a) Principle of fllip for visualizing PIP_3. CFP and YFP, which have different colors, are mutants of green fluorescence protein from *Aequorea victoria* with mammalian codons and additional mutation. Upon binding of PIP_3 with the PHD within fllip, a flip-flop-type conformational change of fllip takes place, which changes the efficiency of FRET from CFP to YFP. b) Schematic representations of domain structures of the present fllips. Shown at the top of each bar are the restriction sites. PHD is derived from human GRP1 (261–382) and selectively binds with PIP_3. PHD–R284C is a mutant PHD, in which the Arg284 is replaced with a cysteine residue so that it does not bind with PIP_3. Ln1, Ln2, and Ln3 are linkers, the amino acid sequences of which are shown at the bottom. MLS1 and MLS2 are membrane localization sequences to the plasma membrane and the endomembranes, respectively, the amino acid sequences of which are also shown at the bottom.[13]

CHEMISTRY
AN ASIAN JOURNAL

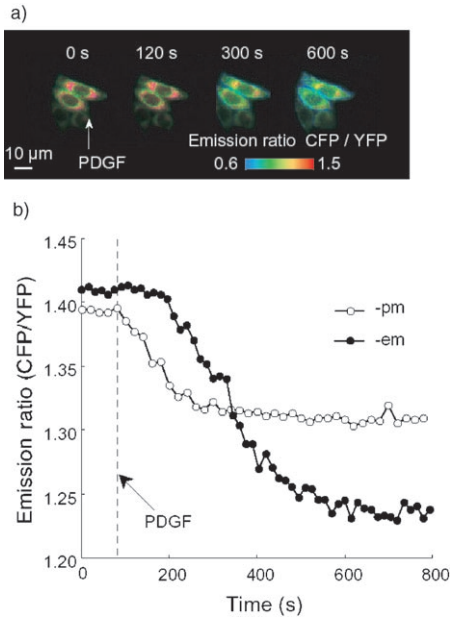


Figure 5. Response of fllip-em to PIP_3 at the endomembranes after PDGF stimulation. a) Pseudocolor images of the CFP/YFP emission ratio before (0 s) and 120, 300, or 600 s after addition of PDGF (50 ng mL^{-1}). Experiments were performed at 25 °C on CHO-PDGFR cells expressing fllip-em. b) Time course of fllip-em and fllip-pm emission ratio after stimulation with PDGF.

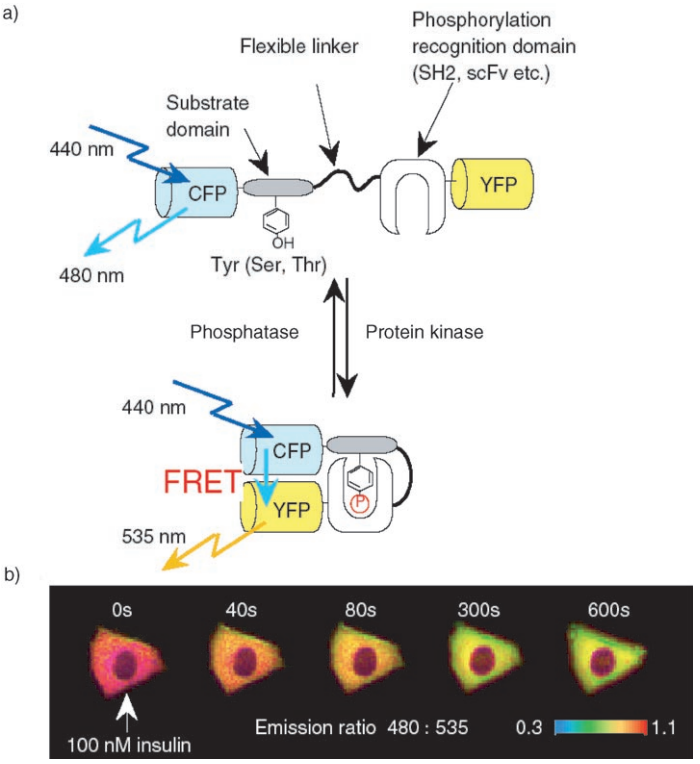


Figure 6. Fluorescent indicators for protein phosphorylation in living cells. a) Principle of phocus for visualizing protein phosphorylation. CFP and YFP are different colored mutants of green fluorescent protein (GFP) derived from *Aequorea victorial* with mammalian codons and additional mutations. b) The CFP/YFP emission ratios are pseudocolor images of the CFP/YFP emission ratios before ($t = 0$ s) and at 40, 80, 300, and 600 s after the addition of 100 nM insulin, obtained from the CHO-IR cells expressing phocus in which a nuclear export signal peptide is attached next to YFP.[14]

causes phosphorylation-dependent changes in the fluorescence emission ratios of the donor and acceptor fluorophores. Upon activation of the phosphatases, the phosphorylated substrate domain is dephosphorylated, and the FRET signal is decreased. We named this indicator "phocus" (a fluorescent indicator for protein phosphorylation that can be custom-made). Not only endogenous domains such as Src homology 2 (SH2) domains, phosphotyrosine binding (PTB) domains, and WW domains are available as the phosphorylation-recognition domain within phocus, but also single-chain antibodies (scFvs) immunized with the phosphorylated substrate sequences of interest. Thus, the present method has more general applicability for kinase signaling in living cells than previously reported fluorescent indicators based on uncontrollable conformational changes in the substrate peptides themselves upon phosphorylation. We used suitable substrate and phosphorylation recognition domains to develop a large number of phocuses for several key protein kinases, including a receptor tyrosine kinase, insulin receptor (1), a serine/threonine protein kinase, Akt/PKB,[15] and a nonreceptor tyrosine kinase, c-Src. Furthermore, these phocuses have been further tailored to visualize the local activity of the respective protein kinases in living cells by fusing appropriate localization sequences/domains with each phocus.[16]

4. Nuclear Receptor/Coactivator 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 interactions between the androgen-receptor ligand-binding domain (AR LBD) and a coactivator protein. This results in an increase in FRET from CFP to YFP (Figure 7). The indicator is capable of distinguishing ligands of different potencies for the AR. The indicator is capable of distinguishing ligands of different potencies for the AR. The present assay is not intended to read out 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 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 the mediation of the actions of drugs for contraception (by PR), inflammation (by GR), and type-2 diabetes (by PPARγ). The present strategy can be used to develop indicators for PR, GR, and PPARγ for screening and characterization of their ligands. The indicators would be helpful in the development of NR-based pharmaceutical drugs against different diseases.

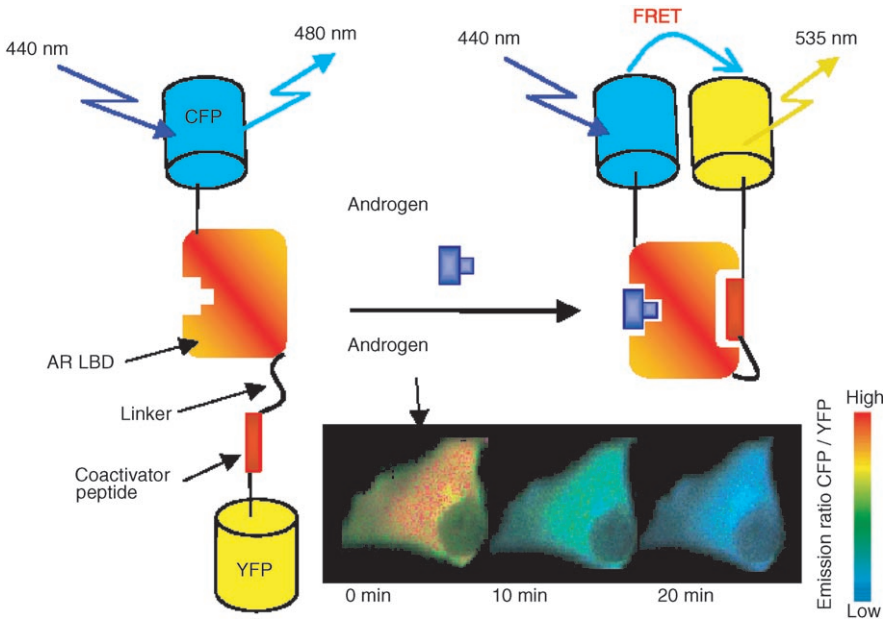


Figure 7. An androgen promotes interaction between the androgen receptor ligand binding domain (AR LBD) and coactivator protein. This results in an increase in the fluorescence resonance energy transfer (FRET) from cyan fluorescent protein (CFP) to yellow fluorescent protein (YFP).[17]

5. Nucleocytoplasmic Trafficking of Functional Proteins

Nucleocytoplasmic trafficking of functional proteins plays a key role in regulating gene expression 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 the 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 carboxy-terminal half of Rluc, which is localized in the nucleus with a fused nuclear localization signal (NLS), and full-length Rluc is reconstituted by protein splicing (Figure 8). The bioluminescence is thereby emitted with coelenterazine as the substrate. The principle of the approach is an extension of the method developed earlier for the identification of mitochondrial proteins.[22]

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 on GFP- or its variant-tagged approach in combination with fluorescence microscopy and computer-driven imaging systems. The systems offer only semiquantitative information, because it is difficult to sort out and distinguish accurately the fluorescence of GFP-tagged proteins localized only in the nucleus in each cell from that left in the cytosol. Furthermore, precision of the observed fluorescence intensities from the nucleus obtained with the statistical analysis is not high, because the number of the cells that can be examined under a fluorescence microscope is limited. In contrast, the present method allows the determination of the subcellular localization of AR is by the luminescence signals generated only when the AR is localized in the nucleus. AR remaining in the cytosol does not induce reconstitution of split Rluc and therefore no background luminescence is observed.

Polychlorinated biphenyls (PCBs) and procymidone have been suspected of causing neurotoxic and antiandrogenic effects, respectively, and possibly adversely influencing the hormonal activities of the brain. We showed the usefulness of the split Rluc reporter for monitoring AR translocation into the nucleus in living mice by implanting the previously mentioned COS-7 cells at a depth of 3 mm in the mouse brain and measuring the emitted bioluminescence with a cooled CCD camera; we thereby investigated the distribution of the chemicals in the brain of living mice. As expected, 2 h after intraperitoneal injection of PCB or procymidone, both chemicals were found to completely inhibit the DHT-stimulated translocation of AR when coelenterazine was injected intracerebrally (Figure 9). From the results, it was concluded that PCB and procymidone can pass through the blood–brain barrier in 2 h to reach the brain and inhibit the AR signal transduction in the organ.[24]

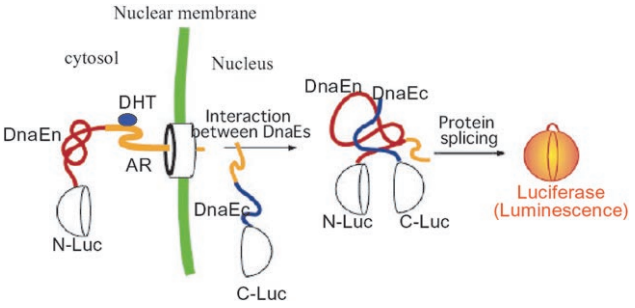


Figure 8. When AR is bound to 5α-dihydrotestosterone (DHT), it translocates into the nucleus, and brings the N- and C-terminal halves of DnaEs close enough to fold correctly, thereby initiating protein splicing to link the concomitant Rluc halves with a peptide bond. The C-terminal half of split Rluc was located beforehand in the nucleus by a fused nuclear localization signal. The cells containing this reconstituted Rluc allow one to monitor nuclear translocation of AR with its luminescence by coelenterazine as the substrate.[24]

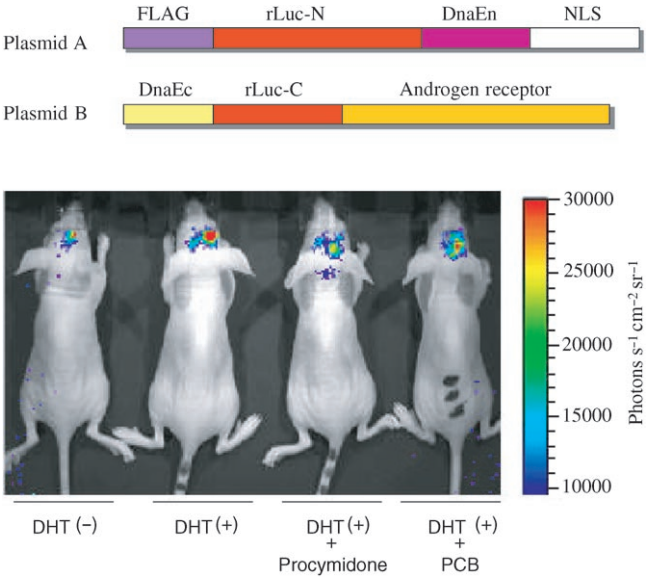


Figure 9. Effects of inhibitors on AR translocation into the nucleus in the mouse brain. PCB and procymidone were found to pass through the blood–brain barrier to reach the brain and inhibit AR signal transduction in the organ. Experiments: 1) $t=0$: COS-7 cells transiently cotransfected with pcRDn–NLS and pcDRc–AR were implanted in the brain of mice. Soon after the implantation, 100 μL of procymidone and PCB were injected intraperitoneally. 2) $t=1$ h: DHT (100 μL) was injected intraperitoneally. 3) $t=3$ h: Coelenterazine was injected intracerebrally.

Similar genetically encoded bioluminescent probes were developed for illuminating protein nuclear transport induced by phosphorylation or by proteolysis.[25] A genetically encoded stress indicator was also reported for the noninvasive imaging of endogenous corticosterone in living mice.[26]

6. Conclusions and Perspectives

To see what was unseen in live cells, fluorescent and/or bioluminescent optical probes have been developed extensively as a core technology for chemistry and biology.

1. Since NO was found to be an endothelium-derived relaxing factor in 1987, it is now well established that NO is a ubiquitous messenger not only for vascular homeostasis but also for neurotransmission and immune systems. Several organic fluorescent indicators have already been developed for the bioimaging of NO. However, these organic dyes covalently react with NO^+ but not with NO radicals in the presence of dioxygen and are therefore not reversible sensors for NO. The organic dyes easily accumulate in subcellular membranes and emit fluorescence signals there in an NO-independent manner. This membrane accumulation of the dyes substantially increases background signals and interferes with the detection of physiologically low concentration of NO in living cells. It is believed that cellular NO must be present in nanomolar concentrations to be physiologically important for exerting its action. A genetically encoded fluorescent indicator for NO described in Section 2.1 reversibly detects NO with high sensitivity (detection limit of 0.1 nM) and allows the visualization of the nanomolar dynamics of NO in single living cells.
2. The lipid second messenger fllips described in Section 2.2 has general applicability for other lipid second messengers. Fllips have two key components, the PHD and the MLS, which can be tailor-made. Lipid second messengers other than PIP_3, such as diacylglycerol and phosphatidylinositol-3-phosphate, could be selectively detected through the use of appropriate binding domains instead of the PHD. Furthermore, by connecting each specific MLS, fllips could be directed not only to the plasma membrane and endomembranes, but also to other organelle membranes, such as the nuclear inner membrane and the outer membrane of mitochondria.
3. A general method was described for the visualization of protein-phosphorylation-based signal transduction in living cells, for example, Akt, Erk and c-Src signaling pathways. The method may be applicable to any protein kinases of interest by changing the substrate sequence, phosphorylation-recognition domain, and localization domain. The use of substrate sequences selectively phosphorylated by kinases of interest can rule out the possibility that such tailor-made indicators are also phosphorylated by other kinases. The indicators should be available for continuous monitoring of protein phosphorylation in tissues and organs of interest in transgenic animals expressing the present indicators. The method is advantageous not only for imaging kinase signaling in single living cells and tissues with high spatial and temporal resolution, but also for multicell analysis aimed at high-throughput screening of pharmaceuticals that regulate kinases and phosphatases.
4. The phenomenon of AR LBD coactivator motif interaction is of prime importance to discriminate between androgen agonist and antagonist ligands. High-throughput screening of a large number of androgenlike compounds, including medicinal drugs and environmental and industrial chemicals, is possible by using the present fluorescent indicator. The approach described herein can be applied to develop biosensors for other hormone receptors such as estrogen, progesterone, thyroid, glucocorticoid, and orphan receptors.
5. A method was also illustrated for the quantitative analysis of the extent of AR translocation with various exo- and endogenous chemical compounds in vitro and inhibitory effects of these chemical compounds on the translocation of AR in the brain of living mice by using a noninvasive imaging technique. This method was exemplified only with AR translocation. The approach could well be applied to any protein translocating from the cytosol to the nucleus, and a rapid screening system could then be developed to discovering novel anticancer drugs or to test chemicals for their adverse effects.

Acknowledgements

This work was supported by the Japan Science and Technology Agency (JST) and the Japan Society for the Promotion of Science (JSPS).

[1] G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **1985**, *260*, 3440–3450.
[2] H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirin, H. Nagoshi, Y. Hirata, T. Nagano, *Anal. Chem.* **1998**, *70*, 2446–2453.
[3] H. Komatsu, N. Iwasawa, D. Citterio, Y. Suzuki, T. Kubota, K. Tokuno, Y. Kitamura, K. Oka, K. Suzuki, *J. Am. Chem. Soc.* **2004**, *126*,16353–16360.
[4] T. Hirano, K. Kikuchi, Y. Urano, T. Higuchi, T. Nagano, *Angew. Chem. Int. Ed.* **2000**, *39*, 1052–1054; *Angew. Chem.* **2000**, *112*, 1094–1096.
[5] *Green Fluorescent Protein* (Eds.: M. Chalfie, S. Kain), Wiley-Liss, New York, **1998**.
[6] D. A. Zacharias, G. S. Baird, R. Y. Tsien, *Curr. Opin. Neurobiol.* **2000**, *10*. 416–421.
[7] A. Miyawaki, J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams, M. Ikura, R. Y. Tsien, *Nature* **1997**, *388*, 882–887.
[8] M. Zaccolo, F. D. Giorgi, C. Y. Cho, L. Feng, T. Knapp, P. A. Negulescu, S. S. Taylor, R. Y. Tsien, T. A. Pozzan, *Nat. Cell Biol.* **1999**, *2*, 25–29.
[9] M. Sato, N. Hida and Y. Umezawa, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 14515–14520.
[10] K. Hirose, S. Kadowaki, M. Tanabe, H. Takeshima, M. Iino, *Science* **1999**, *284*, 1527–1530.
[11] M. Sato, Y. Ueda, M. Shibuya and Y. Umezawa, *Anal. Chem.* **2005**, *77*, 4751–4758.
[12] M. Sato, N. Hida, T. Ozawa, Y. Umezawa, *Anal. Chem.* **2000**, *72*, 5918–5924.
[13] M. Sato, Y. Ueda, T. Takagi, Y. Umezawa, *Nat. Cell Biol.* **2003**, *5*, 1016–1022.
[14] M. Sato, T. Ozawa, K. Inukai, T. Asano, Y. Umezawa, *Nat. Biotechnol.* **2002**, *20*, 287–294.
[15] K. Sasaki, M. Sato, Y. Umezawa, *J. Biol. Chem.* **2003**, *278*, 30945–30951.
[16] M. Sato, Y. Umezawa, *Methods* **2004**, *32*, 451–455.
[17] M. Awais, M. Sato, X. Lee and Y. Umezawa, *Angew. Chem. Int. Ed.* **2006**, *45*, 2707–2712; *Angew. Chem.* **2006**, *118*, 2773–2778.
[18] T. Ozawa, S. Nogami, M. Sato, Y. Ohya, Y. Umezawa, *Anal. Chem.* **2000**, *72*, 5151–5157.
[19] T. Ozawa, A. Kaihara, M. Sato, K. Tachihara, Y. Umezawa, *Anal. Chem.* **2001**, *73*, 2516–2521.
[20] A. Kaihara, Y. Kawai, M. Sato, T. Ozawa, Y. Umezawa, *Anal. Chem.* **2003**, *75*, 4176–4181.
[21] A. Kanno, T. Ozawa and Y. Umezawa, *Anal. Chem.* **2006**, *78*, 556–560.
[22] T. Ozawa, Y. Sako, M. Sato, T. Kitamura, Y. Umezawa, *Nat. Biotechnol.* **2003**, *21*, 287–293.
[23] T. Ozawa, K. Nishitani, Y. Sako and Y. Umezawa, *Nucleic Acids Res.* **2005**, *33*, e34.
[24] S. B. Kim, T. Ozawa, S. Watanabe, Y. Umezawa, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11542–11547.
[25] S. B. Kim, R. Takao, T. Ozawa and Y. Umezawa, *Anal. Chem.* **2005**, *77*, 6928–6934.
[26] S. B. Kim, T. Ozawa and Y. Umezawa, *Anal. Chem.* **2005**, *77*, 6588–6593.

Received: March 9, 2006

