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Accounts

Probing Chemical Processes in Living Cells: Applications for Assay and Screening of Chemicals that Disrupt Cellular Signaling Pathways

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Methods of analysis for bioactive substances that are based on cellular signaling pathways are discussed and compared with the binding assay and bioassay. For nondestructive analysis of chemical processes in living cells, we have developed some general methods and new intracellular fluorescent probes for detecting 1) second messengers, cGMP, diacylglycerol and phosphatidylinositol-3,4,5-triphosphate, 2) protein phosphorylation, 3) protein conformational changes, and 4) protein-protein interactions in live cells under a confocal laser microscope. The approaches for the present probe developments are use of fluorescence resonance energy transfer for reporting binding of substrates (analytes) to molecular recognition domains in dual-fluorophore conjugated probe molecules, and use of protein splicing chemistry for detecting protein-protein interactions. Key molecules and steps of cellular signaling pathways were visualized in relevant live cells using developed fluorescent probe molecules. These probes were found of general importance not only for fundamental biology studies, but also for assay and screening methods for chemicals that inhibit or facilitate cellular signaling pathways. Changes in cellular signals were thereby observed in nongenomic pathways of steroid hormones upon treatment of the target cells with steroid hormones and xenoestrogens. This method of analysis appears to be a rational approach to high-throughput prescreening of biohazardous chemicals such as endocrine disrupting chemicals that disrupt these cellular signaling pathways.

In analytical chemistry, the pursuit of sensitivity, selectivity, precision and accuracy has been *a priori* praised and rewarded. Among these parameters, selectivity in analysis has a unique position. In most chemical methods of analysis, selectivity for analytes against interfering substances is essentially governed by the competitive binding constants between the analyte and its molecular recognition reagent. This is generally the basis for binding assays, such as immunoassays. As a result, chemical methods of analysis owe much to natural bioreceptors, as well as to group reagents, chelating agents, and various supramolecular receptor molecules developed by organic chemistry.

In the case of the analysis of bioactive substances, binding assays are typically used. Conventional binding assays can neither discriminate agonists from antagonists nor give sufficient information on their physiological activities. The physical methods such as NMR and MS cannot provide this informaiton either. The bioassay using intact biological tissue or whole bodies has a unique position in analysis, because it can target bioactive substances. However, the bioassay cannot give molecular level information because of its 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 the cellular mechanism. 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 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.[1–16]

Probing Cellular Signaling Pathways in Living Cells

For nondestructive analysis of chemical processes in living cells, fluorescent probe molecules have been developed for second messengers Ca^{2+} and cAMP, and for ions and small molecules such as Na^+, K^+, Zn^{2+}, Mg^{2+}, Cl^- and NO. In addition, 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.[17]

However, many intracellular chemical processes—cellular

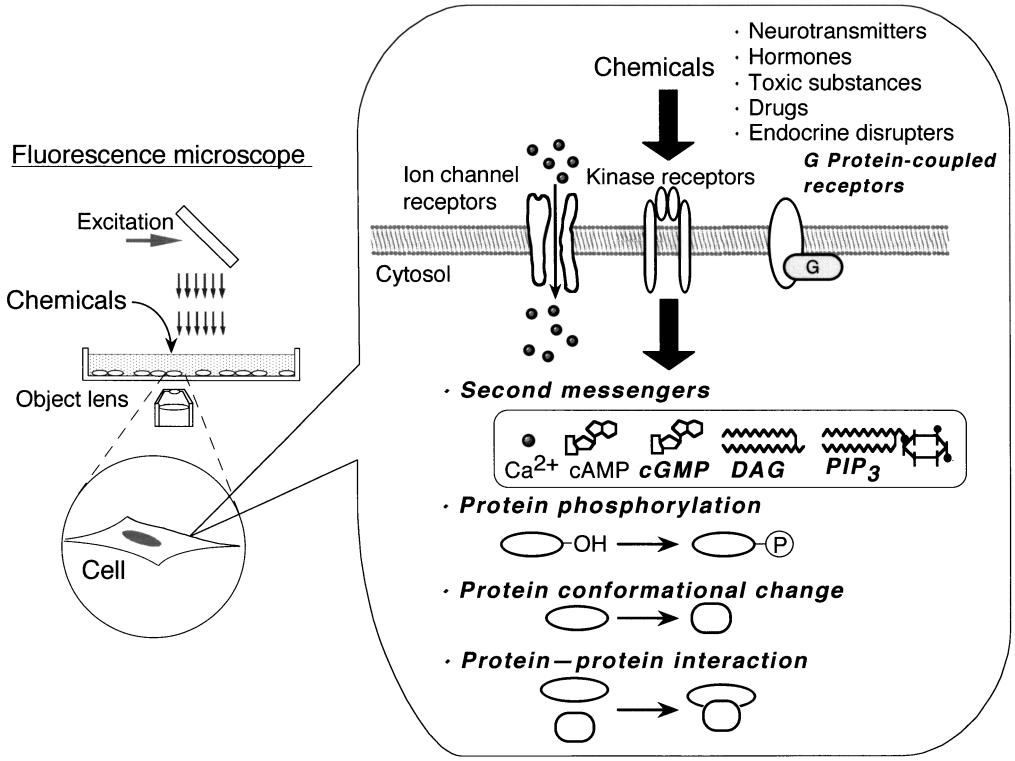

Fig. 1. Probing intracellular signal pathways in live cells under a confocal microscope.

signaling processes are studied still basically by relying on destructive analysis by disrupting hundred 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 now required.

Intercellular signaling substances (first messengers) include neurotransmitters, cytokines and hormones functioning in nerve, immune and endocrine systems. These substances bind either to ion-channel, kinase or G protein-coupled membrane receptor proteins, and trigger the respective down-stream intracellular signaling.

Related to this intracellular signaling, we have developed some general methods and new intracellular fluorescent probes for detecting 1) second messengers, cGMP, diacylglycerol (DAG), and phosphatidylinositol-3,4,5-triphosphate (PIP_3), 2) protein phosphorylation, 3) protein conformational change (kinases etc.); and 4) protein–protein interactions, in live cells under a confocal laser fluorescence microscope (Fig. 1). A method for detecting activation for G protein-coupled receptors is also being explored at the moment.

The developed probes for each particular signaling pathway, (1)–(4), have revealed physiologically relevant response to respective ligands and agonists in live target cells.

The approach for the present probe developments is twofold: 1) use of fluorescence resonance energy transfer (FRET) for reporting binding of substrates (analytes) to molecular recognition domains in dual-fluorophore conjugated probe molecules, and 2) use of protein splicing chemistry for detecting protein–protein interactions. The methods for the probes 1) to 4) are generally applicable to any cells upon being tailor-made in target cells.

Therefore, these probes are of general importance not only for fundamental study of biology, but also for assay and screening methods for chemicals that inhibit or facilitate cellular signaling pathways.

The FRET-type probe molecules developed in our laboratory are all genetically encoded. To construct each fluorescent indicator, two functionally independent parts were chosen and conjugated to each other: one part is for selective molecular recognition for analytes (ligand and agonists), and the other is for transducing the molecular recognition event to the generation or change in fluorescent signals.

On the basis of previously studied structural information in which ligand-induced conformational changes were revealed, the receptor, molecular recognition part, was chosen. As a transducer to detect the ligand (analyte)-dependent conformational change in the receptor part, blue- and red-shifted mutants of green fluorescent proteins (GFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), were fused respectively to both terminal ends of the receptor.

As selective molecular recognition parts, endogenous receptor molecules in each cellular signaling pathway were chosen, CFP and YFP serve as the donor and acceptor fluorophores for fluorescence resonance energy transfer (FRET), of which the efficiency is a function of the proximity (r) and the relative angular orientation (κ) between the two fluorophores, as given in the following equations.[18]

$$E = 1/(1 + r^6 / R_0^6) \quad (1)$$

$$R_0 = (J\kappa^2 Q_0 n^{-4})^{1/6} \times 9.7 \times 10^3 \text{ (in Å)} \quad (2)$$

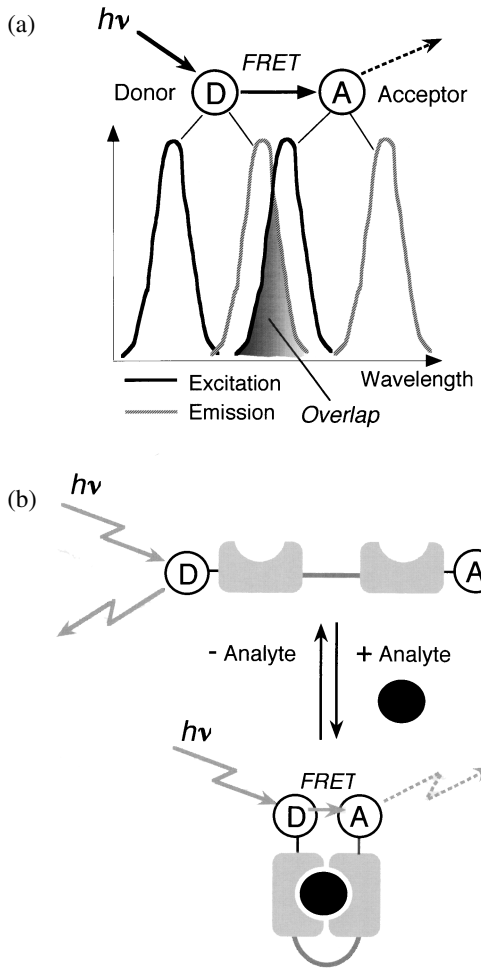

Fig. 2. Principle of FRET used for reporting analyte-binding-dependent structural change of a receptor molecule. See text.

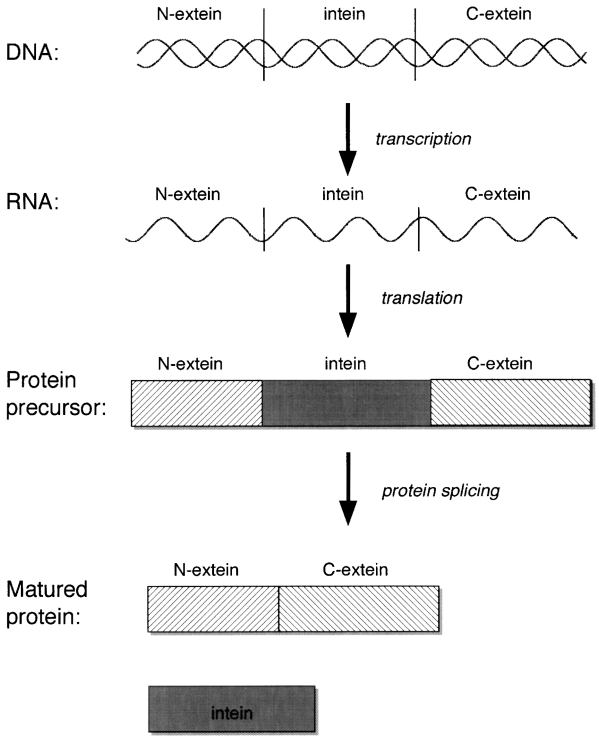

Fig. 3. Posttranslational modification by protein splicing. Genetic information in DNA is directly copied into messenger RNAs (transcription), which in turn directs the biosynthesis of proteins (translation). A specific type of intervening sequence, termed an intein, is excised from an internal site in a precursor protein and the surrounding polypeptide (exteins) are ligated to form the matured protein.

where E is the FRET efficiency, r the actual distance between the center of the fluorophores, R_0 the distance (Å) at which FRET is 50% efficient, Q_0 the quantum yield of fluorescence of the donor in the absence of the acceptor, J the spectral overlap of the donor emission and acceptor absorption, κ^2 the orientation factor for the dipole–dipole interaction, and n is the refractive index of the medium between the donor and acceptor (Fig. 2a).

The agonist (analyte) is expected to alter the r and κ values upon its binding to a receptor that is located between the two fluorophores. The change in the FRET parameters is expected to cause agonist (analyte)-dependent changes in the observed fluorescence spectra, which provide a selective and sensitive measure for intracellular concentration of analytes (Fig. 2b).

Protein biosynthesis was initially thought to be a simple process in which the genetic information in DNA was directly copied into messenger RNAs, which in turn directed the biosynthesis of proteins. But an unexpected discovery was made by two groups independently in 1990 that, in *Saccharomyces cerevisiae*, a nascent 120-kDa translational product of VMA1 gene autocatalytically excised out a 50-kDa site-specific endonuclease (VMA1-derived endonuclease; VDE, also called PI-*Sce*I) and spliced the two external polypeptides to form a 70-kDa catalytic subunit of vacuolar H^+-ATPase.[19,20] This discovery led them to conclude that post-translational removal of polypeptide segments can occur by protein splicing (Fig. 3). The protein splicing is a multi-step processing event involving precise excision of an internal protein segment (intein) from a primary translation product with concomitant ligation of the flanking sequences (extein).[21,22]

The principle for detecting protein–protein interactions is based on reconstitution of a functional protein by the protein splicing reaction.[23,24] As to the functional protein, split-enhanced green fluorescent protein (EGFP) was chosen. The structure of EGFP is composed of eleven strands of β-sheet that form an anti-parallel barrel with short α-helixes forming lids on each end.[25] The fluorescent active center of EGFP is located inside the barrel. In using EGFP, we were initially not sure whether split EGFPs really connect to each other by protein splicing and the ligated EGFP reconstitutes the barrel structure to form its fluorophore. To ascertain this, we performed the following pilot experiment before demonstrating the utilization of protein splicing as a tool for detecting protein–protein interactions. The EGFP was dissected at the position between 128 and 129, that locates at the end of the sixth β-sheet strand of EGFP, and VDE was inserted into the position. A single polypeptide encoding the VDE intervening the N- and

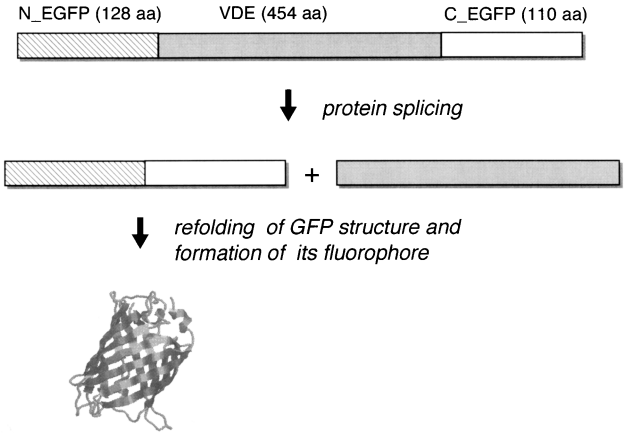

Fig. 4. Reconstitution of split EGFP by protein splicing. A single polypeptide, that is composed of 128 from the N-terminal half of EGFP, 454 residues from VDE and 110 residues from the C-terminal half of EGFP, undergoes protein splicing and thereby N- and C-terminal fragments of EGFP ligate by a peptide bond. The matured EGFP thus formed folds correctly and its fluorophore is formed inside the barrel.

C-terminal halves of EGFP was expressed in *E. coli* and its splicing products were analyzed (Fig. 4). The results revealed that the two external regions of the N- and C-terminal halves of EGFP were ligated with a peptide bond by protein splicing and that the ligated EGFP folded correctly to form its fluorophore.

To explore this finding further, we dessected the single polypeptide into two functional splicing parts. Such dissection was achieved by deletion of a functionally unrelated endonuclease motif from a 185–389 amino acid region. The resulting peptide fragments, termed optical probes, are linked to a protein of interest (protein A) and its target protein (protein B) (Fig. 5). When an interaction occurs between the two proteins, the N- and C-terminal halves of VDE are brought in close proximity and undergo correct folding, which induces the splicing and thereby N- and C-terminal fragments of EGFP directly link to each other by a peptide bond. This reconstitution of EGFP is monitored by its fluorescence at 510 nm. The intensity of the fluorescence is proportional to the number of interacting protein pairs. In our proof of this principle, we attached calmodulin (CaM) and its target peptide, known as M13, derived from skeletal muscle myosine light-chain kinase. Upon coexpression of CaM and M13 connected with the optical probes in *Echelicia coli*, their interaction induced protein splicing, by which split EGFP underwent correct reconstitution and its fluorescence could be monitored.[23,24]

Assay and Screening of Chemicals that Disrupt Cellular Signaling Pathways

To date, the number of synthetic molecules that are potentially harmful to the physiology of animals and plants is more than one hundred thousand, including pesticides and industrial chemicals, and the number continues to increase. One of the central issues regarding these chemicals is their possible endocrine-disrupting effects.

The purpose of analysis of these potential endocrine-disrupting chemicals (EDCs) is twofold: (I) their identification and quantitative analysis, and (II) risk assessment of each EDC candidate with respect to reproductive and developmental problems posed to animals and humans.

Recently, International Union of Pure and Applied Chemistry, IUPAC, made a comprehensive survey of natural and anthropogenic environmental estrogens in relation to their scientific basis for risk assessment.[26] Environmental Protection Agency, EPA,[27] and Organization for Economic Cooperation and Development, OECD,[28] have summarized the methods so far used for risk assessment of EDCs on animals and humans, and have proposed a series of screening tests to identify potential endocrine disruptors. EPA recommends prescreening using binding and reporter gene assays, followed by a secondary screening with TIER1 and the final test with TIER2, both

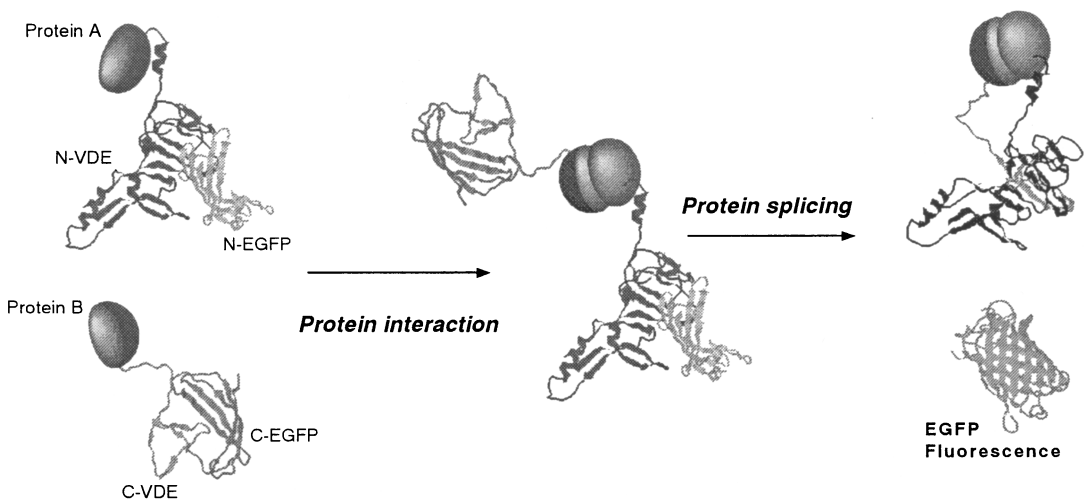

Fig. 5. Proteins of which interactions are being monitored are attached to the N- (blue) and C- (orange) terminal portions of VDE and split EGFP (gray). When the proteins interact, the two portions of VDE come close enough to fold together and initiate protein splicing. The two halves of EGFP are ligated and released.

based on bioassays.[27] OECD also recommends dual approaches, using in vivo animal tests and in vitro screening, basically the same in principle as those of EPA.[28] The initial prescreening should have a high throughput, because the number of synthetic molecules that are potential EDC candidates is large. For this high throughput prescreening (HTPS), α- and β-estrogens and androgen receptors are typically used in both binding and reporter gene assays.[29,30]

The receptor binding assay for estrogens and androgens is based on the competitive binding of these hormones to estrogen and androgen receptors, respectively. The reporter gene assay for steroid hormones is a serial or double binding assay, in which a steroid-hormone-bound steroid receptor is further bound to a promoter region of a gene to form a functional transcription factor that switches on a reporter gene. These two assay methods are now representative of HTPS for the risk assessment of EDC candidates.

The advantage of receptor binding and reporter gene assays is that they are quite simple to perform and allow the identification of all endocrine disrupters that act through the estrogen receptor. However, in the case of the binding assay, the binding selectivity does not necessarily represent the expected physiologically relevant selectivity. Not only can antagonists and agonists not be discriminated, but also the efficacy of agonists will also depend on the extent of increase or decrease in the subsequent cellular signaling. In the reporter gene assay, use of artificial reporter genes naturally results in gene expressions different from those of endogenous ones and in biased output of physiological consequence with respect to risk assessment of EDCs.

Moreover, EDC candidates such as methylmercury and tributyltin, which do not exert effects via nuclear receptors, may not be detectable by receptor binding assay nor by reporter gene assay; that is, targets (receptors) of synthetic molecules may not necessarily be nuclear transcription factors such as estrogen and androgen receptors. EDC candidates that exhibit positive responses in these HTPS are then subject to in vivo bioassays (animal tests) based on TIER1 followed by TIER2.[27]

In view of the above-mentioned circumstances, there is a need to develop a new HTPS method that is in contrast to the conventional receptor binding and reporter gene assays. The key concept of this method is that the measure of risk assessment should be based on the extent of changes in relevant cellular signaling pathways and/or gene expressions upon loading EDCs candidates to target cells. A living target cell is stimulated by EDC candidates and the resulting changes in relevant key steps in cellular signaling pathways are directly measured in vivo using fluorescent probe molecules for cellular signaling pathways. For the purpose of nondestructive analysis of key steps in cellular signaling pathways, we have so far developed fluorescent probe molecules for second messengers such as cyclic GMP and lipid messengers (PIP_3, diacylglycerol), activation of Ca^{2+} signaling, protein phosphorylation, and protein-protein interactions.

Steroid hormones such as estrogens and androgens are known to bind to their receptors, the complexes of which are transported into the nucleus to control the expression of genes. This is the so-called genomic or classical pathway for steroid-hormone signaling.

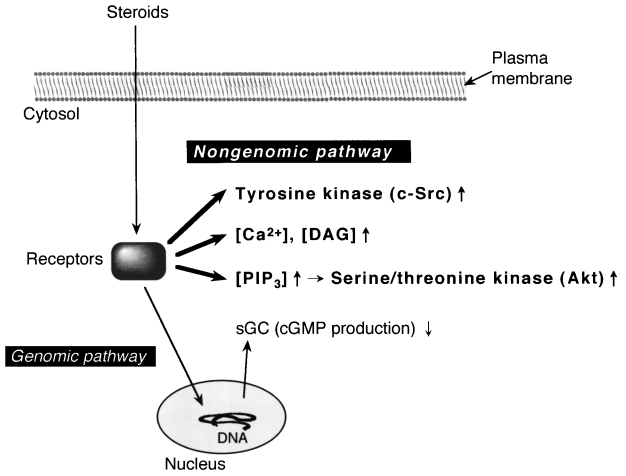

Fig. 6. As regards nongenomic pathways involving estrogen and androgen, estrogen and androgen receptors were induced to interact with cSrc upon their binding with 10 nM 17β-estradiol (E2) or 10 nM androgen in MCF-7 cells.[31] Moreover, in MCF-7 cells and osteoblasts, 100 nM E2 has been reported to increase Ca^{2+} or diacylglycerol concentrations.[32,33] In vascular endothelial cells, 10 nM E2 and 10 nM thyroid hormones increased the PIP_3 concentration and activate serine/threonine kinase Akt.[34] On the other hand, in the rat uterus, the expression of soluble guanylate cyclase was inhibited in 24 hours, via a genomic pathway upon treating with E2 (40 μg/kg body weight).[35]

Recently, nongenomic pathways in intracellular signaling have been found for steroid hormones (Fig. 6). When stimulated with steroid hormones, target cells exhibited rapid intracellular signal changes in several seconds, indicating nongenomic signaling pathways corresponding to those in the cytosol. Receptors for steroid hormones were observed to function not only as transcription factors in the nucleus, but also as cytosolic signaling proteins, such as the direct interaction of tyrosine kinase (cSrc) with some target proteins, resulting in a change in cytosolic concentration levels of second messengers such as Ca^{2+} and PIP_3.

cGMP. It was found in 2001 that 17β-estradiol (E2)-loaded rat uterus exhibited a marked decrease in the expression level of soluble guanylate cyclase (sGC) that produces cGMP.[35] We also observed in human uterine smooth muscle tumor cells (SKN), using reverse transcription PCR, that the expression levels of both α and β subunits of sGC decreased in 24 hours in an E2 concentration-dependent manner. Interestingly, representative EDCs such as bisphenol-A, genistein, and DES did not exhibit any inhibitory effect on β subunit expression compared with E2. In contrast, α subunit expression was more decreased by bisphenol-A than by E2.

In human SKN cells expressed with our fluorescent indicator CGY for visualizing cGMP[36] (Fig. 7), we further found these E2-and bisphenol-A-dependent decrease in the sGC expression level as a decrease in NO-induced cGMP generation observed under a fluorescence microscope as shown in Fig. 8. Other representative EDCs, such as genistein, and DES, were also tested. This approach does not rely on exogenous reporter genes such as luciferase; thus endogenous sGC generation was

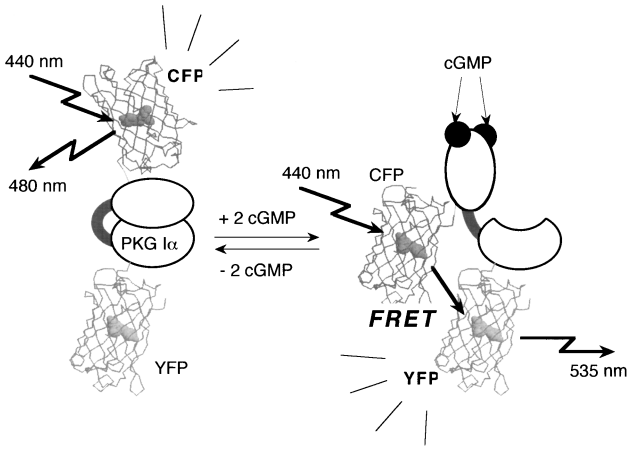

Fig. 7. A fluorescent probe molecule for cGMP. cGMP-dependent protein kinase (PKGIα) is used for selective recognition of cGMP. PKGIα is structurally changed upon binding with cGMP, which is monitored by fluorescence resonance energy transfer (FRET) between two color mutants of GFPs, CFP and YFP, attached to N- and C-terminals of PKGIα respectively. This probe is called CGY after its molecular structure consisting of CFP, PKGIα and YFP, and used to measure cGMP levels in living cells.[36]

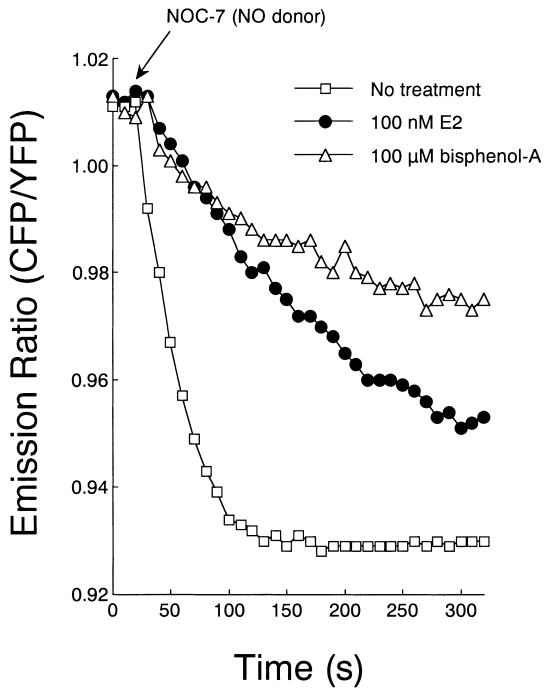

Fig. 8. Imaging the inhibitory effect on sGC expression of E2 and bisphenol-A. After treating the SKN cells expressing the cGMP indicator, CGY, with 100 nM E2 or 100 μM bisphenol-A for 4 hours, NO-stimulated cGMP generation was visualized in the single living cells under a fluorescence microscope.

monitored instead. This method may therefore become a more physiologically relevant "reporter gene assay" for the risk assessment of environmental estrogens.[37] This would be a kind of biomarker.

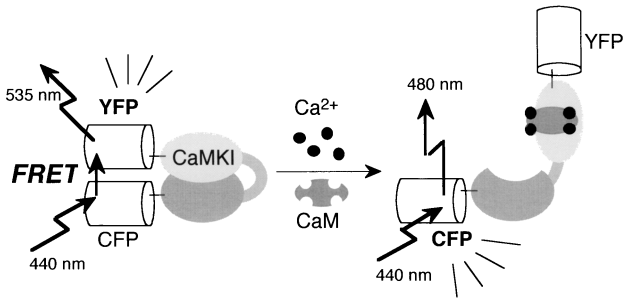

Fig. 9. A fluorescent probe for monitoring intracellular calmodulin dependent protein kinase (CaMK) activity.

Calmodulin-Dependent Protein Kinase (CaMK). Like many peptide hormones and neurotransmitters, 17β-estradiol (E2) was found to change intracellular Ca^{2+} signals in a nongenomic manner, using an intracellular fluorescent Ca^{2+} probe. However, for some twenty of Ca^{2+} signaling molecules including kinases and phosphatases, we do not yet know when, where, and to what extent they are activated and functioning in a Ca^{2+}-dependent manner. We therefore developed a fluorescent probe for imaging of activation of Ca^{2+}-dependent serine/threonine kinase CaMK. This probe is a fusion protein consisting of CaMK that is inserted between two differently colored GFP variants: CFP and YFP. This molecule has been confirmed to retain the expected characteristics of wild-type CaMK in cells including its phosphorylation and structural change upon interaction with Ca^{2+}/calmodulin. This molecule emits a fluorescent (FRET) signal in vivo, which reflects the extent of activation of CaMK itself (Fig. 9).[38]

In estrogen-responsive human breast cancer cells (MCF-7), this CaMK-activation probe was expressed; the stimulation with 100 μM ATP led to CaMK activation, while 100 nM E2 did not cause CaMK activation. This indicates that E2 affects in a nongenomic manner to elevate the intracellular Ca^{2+} level as determined by a Ca^{2+} fluorescent indicator, but that the elevated Ca^{2+} concentration is not sufficient to activate the downstream CaMK route. On the other hand, 100 μM bisphenol-A stimulated more amplified and prolonged Ca^{2+} transients compared with E2, resulting in transient and reproducible activation of CaMK. Other EDCs, e.g., 10 μM nonylphenol and 1 μM DES, also stimulated more amplified Ca^{2+} transients compared with E2, while surprisingly, these EDCs did not cause any significant transients of CaMK activation. These results exemplify the significance of serial analysis of signaling events for screening of endocrine disrupters. Moreover, other Ca^{2+} signaling routes are currently being examined.

Protein Phosphorylation. Cellular signaling via protein phosphorylation cannot be missed during HTPS of EDCs. A general approach to direct detection of protein phosphorylation in live cells has been developed.[39] Custom-made fluorescent probes for protein phosphorylation, designated as phocus, can be designed and constructed for any given pairs of phosphorylation recognition and substrate domains. As shown in Fig. 10, a substrate domain of interest and a corresponding phosphorylation recognition domain are connected with a flexible linker peptide. CFP and YFP are attached as the flanking pieces of the probe protein. Upon phosphorylation of the substrate do-

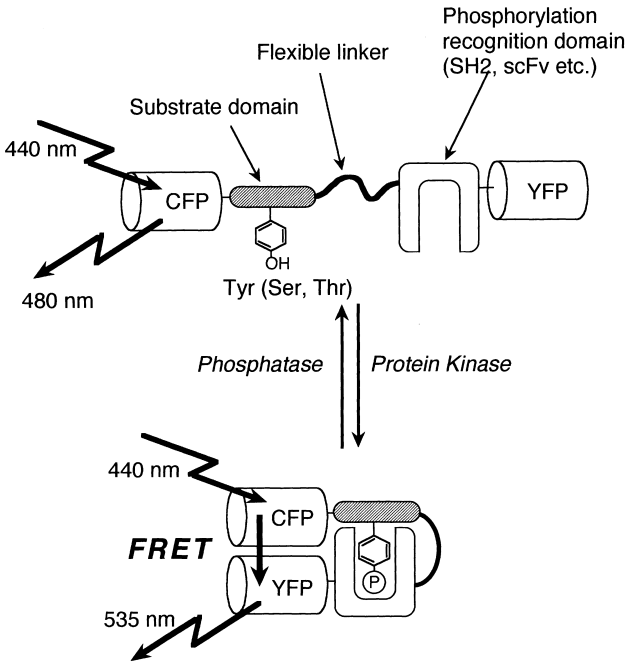

Fig. 10. Principle of phocus for visualizing protein phosphorylation in living cells. CFP and YFP are different color mutants of green fluorescent protein (GFP) derived from *Aequorea victoria* with mammalian codons and the following additional mutations, CFP and YFP. Upon phosphorylation of the substrate domain within phocus by protein kinase, the adjacent phosphorylation recognition domain binds with the phosphorylated substrate domain, which changes the efficiency of fluorescence resonance energy transfer (FRET) between the GFP mutants within phocus.[39]

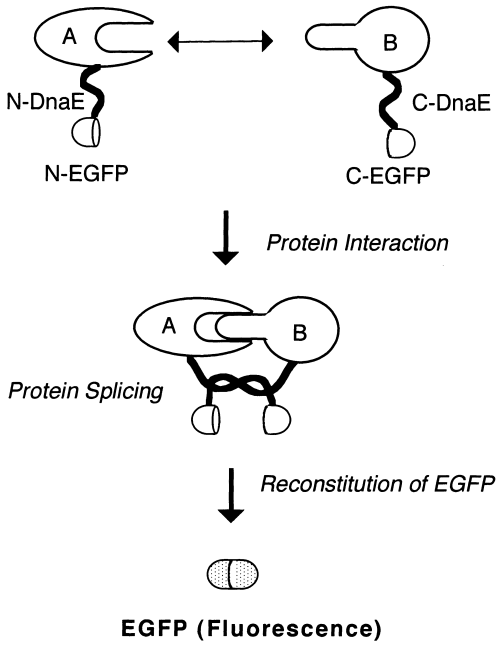

Fig. 11. Release of fluorescent protein GFP reveals target protein–protein interactions.[42]

main, the phosphorylation recognition domain is bound to the phosphorylated substrate, which results in a large structural change in the molecule, and generation of FRET.

The working principle of this probe molecule was confirmed with an insulin signaling system for quantitative imaging of tyrosine phosphorylation of an involved protein in living CHO-IR cells.[39]

This phocus (Fig. 10)[39] was further tailored for detecting protein phosphorylation in nongenomic estrogen signaling pathways for use for EDCs screening. To observe estrogen-induced activation of tyrosine kinase cSrc and serine/threonine kinase AKT/PKB, Src-phocus and Akt-phocus were developed, respectively, using suitable pairs of phosphorylation recognition and substrate domains. Now we are in the stage of evaluating the effect of EDCs on these nongenomic kinase signaling pathways.

Protein–Protein Interactions. Protein interactions in eukaryotic cells play requisite roles such as controlling enzymatic activities, gene expressions and protein phosphorylation/dephosphorylation. When a particular cell was stimulated with EDCs, homeostasis of the cellular signaling is disturbed and certain protein interactions may change. By monitoring this change, in particular protein/protein interaction in a living cell, one can evaluate the extent of any possible adverse effects of EDCs. Like the other fluorescent probe molecules described above, it is significant that an HTPS method for EDCs based on protein interactions in living cells be developed.

The conventional approach of destructive analysis for evaluating the protein/protein interaction is not applicable for HTPS, in which the number of EDC candidates is too large for batch-type destructive analysis. In view of these considerations, we have developed a new method for detecting protein-protein interaction based on protein splicing, in which the interactions are visualized in living cells.

In this detection system, VDE was replaced with DnaE derived from Synechocystis, thereby improved sensitivity and screening time were attained. Proteins whose interactions were being monitored are attached to the N- and C-terminal portions of DnaE and the green fluorescent protein (EGFP). When the proteins interact, the two portions of DnaE came sufficiently close together to fold and initiate protein splicing. The two halves of EGFP were ligated and released to fluoresce (Fig. 11).[40–42]

Unlike earlier protein interaction assays, the split-GFP system involves the reconstitution of GFP, and does not require that the protein-protein interactions occur near the cell nucleus and reporter genes or that an enzyme substrate be present. This will make the method generally more useful and will allow screening of the interactions in the cytosol or at the inner membrane level. This GFP splicing system was applied to EDCs screening using living eukaryotic cells. As an example, the interaction between the androgen receptor (AR) and cSrc is demonstrated in Fig. 12a.

The male sex hormone androgen, dihydrotesterone (DHT), has been known to bind the androgen receptor (AR) in the cytosol, and to induce cell proliferation. Furthermore, DHT increased the kinase activity of cSrc located adjacent to the cell membrane in the cytosol of prostate cancer cells and osteoblasts. On the basis of these findings, the N- and C-terminal

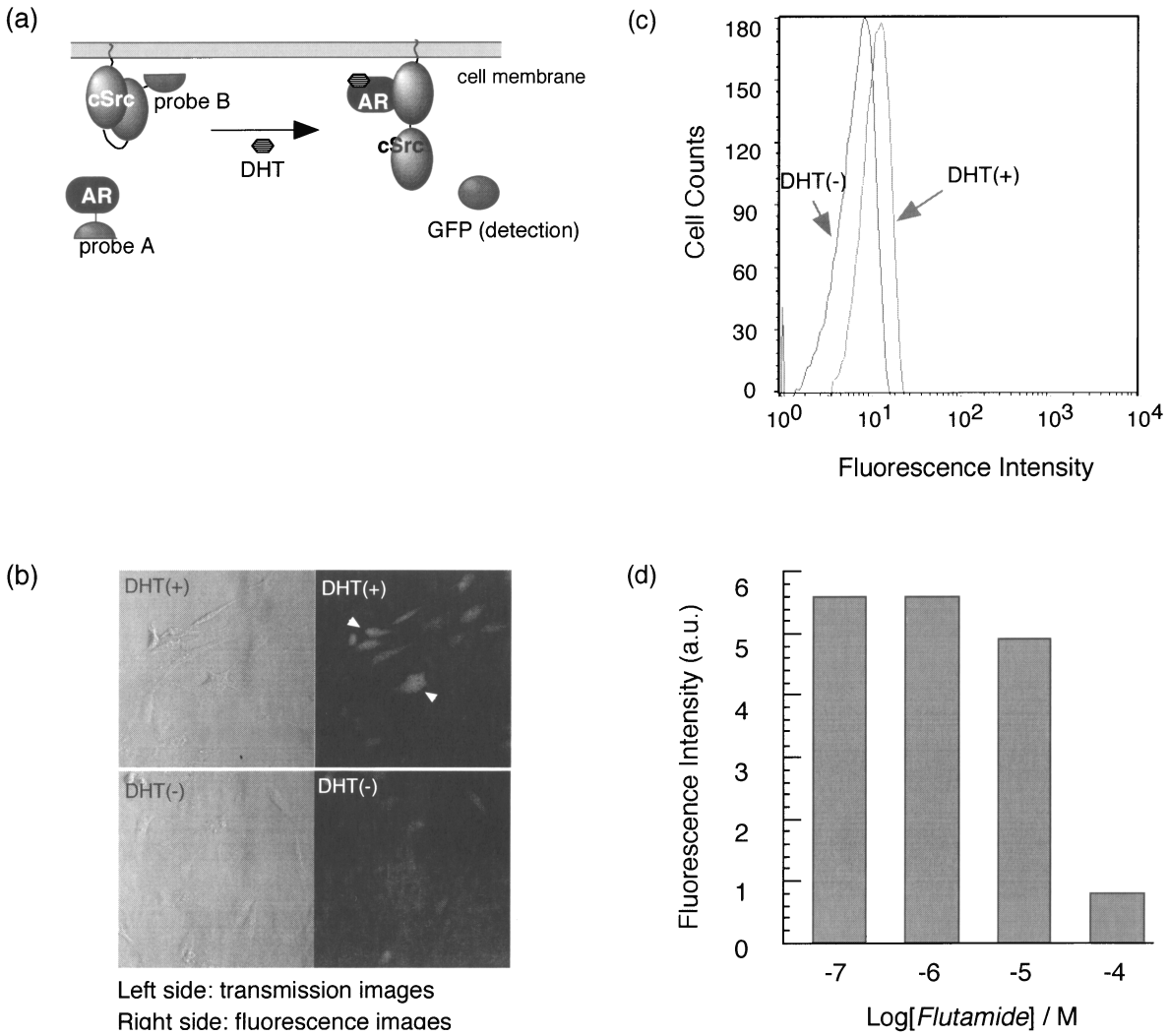

Fig. 12. (a) Detection of androgen hormone induced cSrc-androgen receptor interaction inside the cell membrane. (b) Male sex hormone, dihydrotesterone (DHT) induced this interaction and released GFP to fluoresce. (c) Analysis of cSrc-androgen receptor interaction using the split GFP system. The number of fluorescent cells detected by FACS increases following addition of DHT. (d) Dependence of cSrc-androgen receptor interaction, which was measured with the GFP fluorescence, on the increasing concentration of added flutamide, an AR antagonist.[3]

halves of the split GFP probe were attached to cSrc and AR, respectively, and expressed in NIH3T3 cells.

Upon stimulation with DHT, the cells emitted GFP fluorescence, which indicates that DHT induced the interaction between cSrc and AR inside the cell membrane, and GFP was generated as a result (Fig. 12b). Moreover, flow-cytometry (fluorescence-activated cell sorter, FACS) confirmed that the number of fluorescent cells increased with an increase in the DHT concentration (Fig. 12c).

Furthermore, flutamide, an AR antagonist, decreased the number of fluorescent cells, indicating that cSrc-AR interaction was inhibited by flutamide (Fig. 12d).[43]

The split GFP system can thereby detect certain intracellular protein–protein interactions nondestructively in living cells. This assay system will therefore be used for EDC screening on the basis of a nongenomic pathway involving AR.

Nuclear Imports. Protein trafficking into the nucleus plays an important role for efficient and rapid signal transduction from the plasma membrane to the nucleus. The communication between cytoplasm and nucleus is essential for the maintenance of homeostasis in living cells. Previous studies of intracellular localization of proteins have been performed by immunohistochemistry or immunocytochemistry using specific antibodies, which require fixing and killing the cells. In contrast, GFP that is fused to a protein of interest has enabled analysis of the dynamics of nuclear and cytoplasmic localization in response to different extracellular signals with spatial and temporal resolution. The use of these methods is, however, limited only to qualitative analysis.

The protein splicing system is a powerful approach to quantitatively assess the amount of a protein located in the nucleus. The concept is shown in Fig. 13. Androgen receptor is used as a target; it is attached to the N-terminal half of DnaE and luciferase. The C-terminal half of the DnaE is connected with a short amino acid sequence, referred to as the nuclear localization signal (NLS). The NLS allows the C-terminal probe mol-

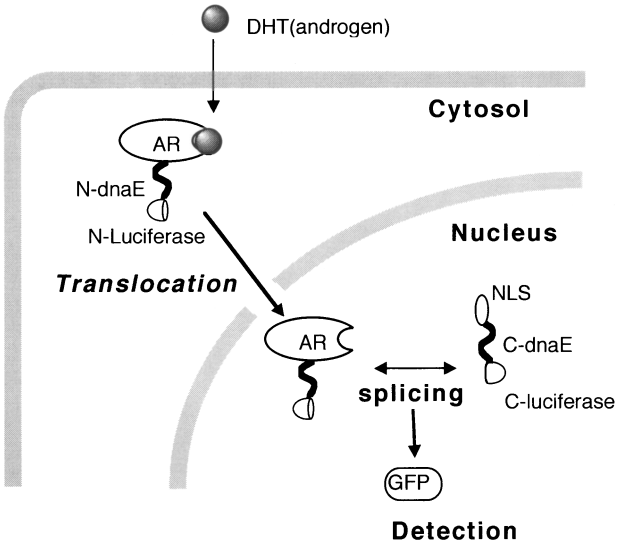

Fig. 13. Putative proteins, of which translocations are to be detected, are attached to the N-terminal portions of DnaE and luciferase. When the proteins translocate to the nucleus, the two portions of DnaE come close enough to fold together and initiate protein splicing. The two halves of the split-luciferase are ligated and released, the amount of which is a quantitative measure of the nuclear import of the protein.

ecule to reside in the nucleus. When the cell is stimulated with androgen, its receptor translocates into the nucleus and approximates the C-terminal probes in the narrow nuclear compartment. Since the N- and C-terminal halves of DnaE have some affinity, luciferase formation following splicing reaction proceeds. The luminescence intensity of thus formed matured luciferase was found to be proportional to the amount of androgen receptor that translocates to the nucleus.[44] There will be numerous other applications in this system to the quantitative detection of a localized protein, such as in the mitochondria or endoplasmic reticulum.

Gene Expressions. Reporter gene assays for estrogen (E2) and environmental estrogens rely on two discrete binding processes of the estrogen receptor (ER); i.e., ER binds to E2, and this ER–E2 complex is transported to the nucleus, where it further binds to the corresponding regulatory sequence of DNA, thereby working as a transcription factor. However, the chosen reporter genes such as luciferase and β-galactosidase inserted near the regulatory sequence are simply artificial.

On the other hand, when estrogen is added to target cells (e.g., MCF-7), how many and what kind of endogenous genes are in fact expressed and to what extent?

Recently developed technologies allow us to survey thousands of gene expressions with respect to the effect of EDCs and synthetic molecules on the expression of relevant genes.

Serial analysis of gene expression, SAGE, has been used to systematically survey the change in expressions of relevant genes in MCF-7 cells, upon addition of estrogen and other environmental estrogens.[45] SAGE is a technique in which sequences of 10 base pairs of human gene expression products, mRNA, each supposed to sufficiently represent a particular gene, are systematically analyzed, and corresponding genes are identified therefrom with reference to the data in gene banks.

In addition to known estrogen-responsive genes such as PS2, cathepsin D, and a highly-mobile protein1, a new estrogen-responsive gene, WISP-2 (Wint-1 inducible signaling pathway protein 2) was identified. The expression of WISP-2 was E2 concentration dependent, and other environmental estrogens including bisphenol-A, genistein, nonylphenol, DES, daizein, and zearalenone also induced WISP-2. Simultaneous addition of an estrogen antagonist ICI182,780 inhibited the expression. The addition of progesterone, dexamethasone, thyroid hormone or 2,3,7,8-TCDD did not induce the WISP-2 gene either. These results indicate that the estrogen receptor was involved in the expression of WISP-2. The WISP-2 protein was in fact detected in estrogen-stimulated MCF-7 cells by western blotting using a polyclonal antibody from WISP-2-immunized rabbit and peptide antibodies against human and mouse WISP-2. This WISP-2 protein was found not only in the cytosol of MCF-7 cells but also in the supernatant of the medium for the cell culture of MCF-7, indicating that it is a secretory protein. Based on these results, WISP-2 may become a biomarker for the risk assessment upon exposure of humans to environmental estrogens. The only known biomarker is vitellogenin, which is employed to probe the extent of exposure of fish to EDCs.

DNA chips or gene chips can in principle search thousands of target mRNAs whose expression levels increase or decrease upon treatment of relevant cells with EDCs. However, the choice of genes to be immobilized on the chip is not straightforward. One possible approach would be to have estrogen represent other EDC candidates that might be able to change gene expression upon treatment of MCF-7 cells with xenoestrogens. The selected genes will be used for the gene arrays on the chip and for screening to determine whether the same series of genes are likewise affected by xenoestrogens. This approach is most effective when the number of genes is large and/or the number of xenoestrogen candidates to be screened is also large.

A disadvantage for limiting the number of genes on the chip is that genes other than those on the chip may be missed when they are expressed only with some particular EDC candidates. A DNA chip with a very comprehensive collection of genes from the human liver tissue and hepatocytes is being constructed for use in EDC screening on the basis of SAGE analysis.[46]

Ideally, the most appropriate approach to construct a DNA chip for any purpose, including that investigated in the present study is to mount all of human genes, the number of which is now believed to be some ten thousands. This is naturally not feasible at the moment, but will become so when all human genes are identified in the foreseeable future.

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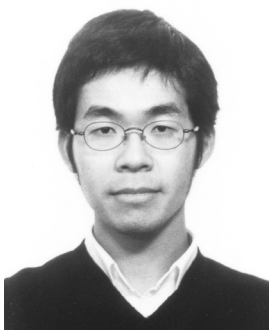

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