

Review Article

Stable expression of FRET biosensors: A new light in cancer research

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The constituents of the oncogene signal transduction pathway are promising targets for anticancer drugs. Despite the wealth of available knowledge regarding their molecular properties, the spatiotemporal regulation of the signaling molecules remains elusive. Biosensors based on the principle of FRET have been developed to visualize the activities of the signaling molecules in living cells. However, difficulties in the development of sensitive FRET biosensors have prevented their widespread use in cancer research. The lack of cell lines constitutively expressing a FRET biosensor has also limited their use. In this review, we will introduce the principle of FRET-based biosensors, describe an optimized backbone of the FRET biosensors, techniques to express FRET biosensors stably in the cells, and discuss the future perspectives of FRET biosensors in cancer research. (*Cancer Sci* 2012; 103: 614–619)

Cancer is a genetic disease caused by alterations in genes that regulate various aspects of cellular functions. For the last quarter of a century, molecular biology and biochemistry have been the two major fields contributing to an understanding of the genes and gene products involved in the development and progression of cancer cells. Consequently, a substantial portion of the existing knowledge was obtained from homogenized cells. For a more comprehensive understanding of the cancer cells, we will need to obtain spatiotemporal information of genes and gene products in living cells and tissues, thus, live cell imaging is the next promising technique in cancer research.

The application of GFP was an important breakthrough for the understanding of the dynamics of gene products in living cells. Green fluorescent protein and other fluorescent proteins have been used to investigate the dynamic localizations of molecules of interest in living cells and animals.⁽¹⁾ Furthermore, based on the principle of FRET, GFP-based genetically encoded biosensors have been developed for the monitoring of activities of signaling molecules.⁽²⁾ For example, with FRET biosensors it has been shown that Ras is preferentially activated at the free edge of the cells⁽³⁾ and that Ras-binding to Raf induces dimerization of Raf.⁽⁴⁾

Previously, we summarized FRET biosensors and the findings obtained using these biosensors in relation to cancer research.⁽⁵⁾ Here, we report an optimized backbone of FRET biosensors that facilitates the development of highly sensitive FRET biosensors, the techniques used to establish cell lines stably expressing FRET biosensors, and discuss how these advances contribute to cancer research. The detailed experimental procedures can be found in our previous review article^(6,7) and are also available on our website (<http://www.lif.kyoto-u.ac.jp/labs/fret/e-phogemon/index.htm>), so we will

limit ourselves to describing the recent progress in FRET biosensor technologies and their application to cancer research.

Monitoring the Activities of Signaling Molecules Using FRET-Based Biosensors

Förster (or fluorescence) resonance energy transfer is a process by which a donor fluorophore in an excited state non-radiatively transfers its energy to a neighboring acceptor fluorophore, thereby causing the acceptor to emit fluorescence at its characteristic wavelength.⁽⁸⁾ The FRET efficiency depends on several factors: (i) a proper spectral overlap between the donor and the acceptor; (ii) the distance between the two fluorophores; and (iii) the relative orientation of donor and acceptor (Fig. 1A–C). The last two factors are particularly important for the development of FRET biosensors with high sensitivity.^(8,9)

The FRET biosensors generally comprise an acceptor fluorophore, a donor fluorophore, a ligand domain, a sensor domain, and linkers that connect each domain (Fig. 1D). The sensor domain is designated as such because it senses the signal and changes its conformation. Phosphorylation, GTP loading, and phospholipid binding are representative signals that induce the conformational change of the sensor domain. The ligand domain binds to the conformation-changed sensor domain, thereby bringing the acceptor and donor in close proximity to evoke FRET. The fluorophores could be either organic chemicals or fluorescent proteins; however, here we restrict the description only to the fluorescent proteins for the sake of brevity.

The FRET biosensors are classified into intermolecular (or bimolecular) and intramolecular (or unimolecular) types (Fig. 1D).⁽¹⁰⁾ Intermolecular FRET biosensors consists of two molecules, one comprised of an acceptor fluorophore and the ligand (or sensor) domain and the other comprised of a donor fluorophore and the sensor (or ligand) domain. The intermolecular biosensors are particularly useful for detecting protein–protein interaction within the cells. However, for the quantification of FRET, careful correction of bleed-through of donor fluorescence into the FRET channel and the cross-excitation of acceptor fluorophores is essential, making it difficult to use intermolecular FRET biosensors for routine applications.⁽⁴⁾

Intramolecular FRET biosensors combine all components into a single molecule. In contrast to the intermolecular type, the use of intramolecular FRET biosensors is straightforward. The expression of the FRET biosensor and the ratio-imaging of donor and acceptor fluorescence are sufficient to obtain the FRET image. However, the development of intramolecular

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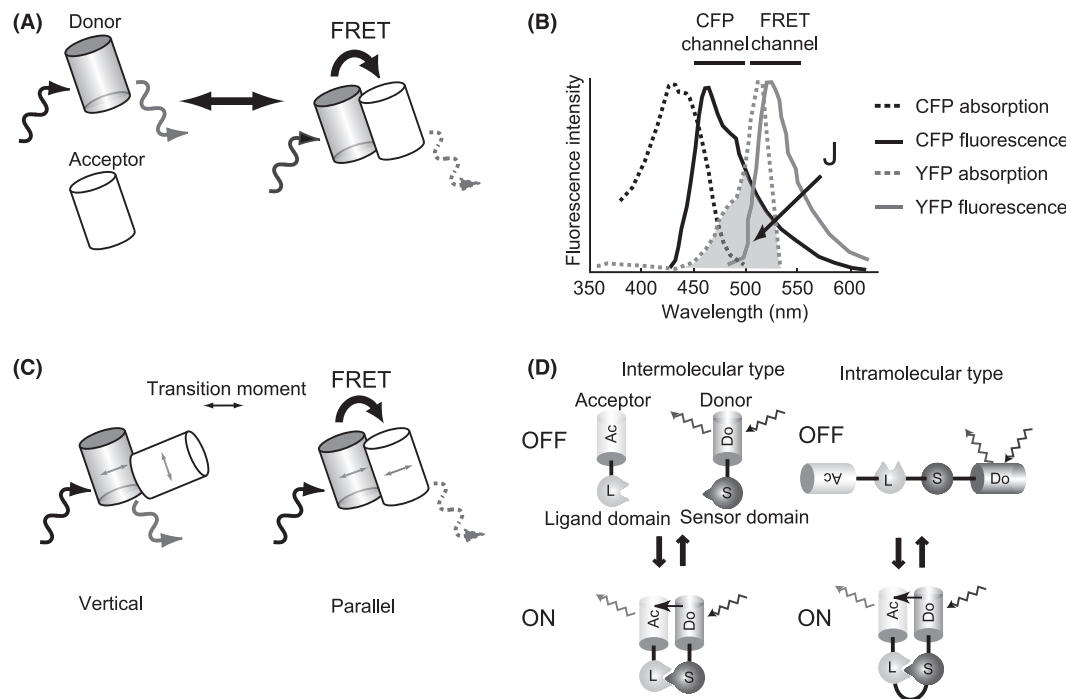


Fig. 1. Three major parameters that determine FRET efficiency and the two types of FRET biosensor. (A) The distance between the donor and acceptor fluorophores is critical. The distance that gives the half maximal is named the Förster distance. In the case of GFP-based FRET, the Förster distance ranges from 3 to 5 nm. Note that the diameter of GFP is ca. 4 nm. (B) Normalized excitation and fluorescence spectra of donor and acceptor fluorophores are shown. The donor fluorescence spectrum should overlap with the acceptor absorbance spectrum. The larger the overlapped area (J), the higher the FRET efficiency. CFP, cyan-emitting fluorescent protein; YFP, yellow-emitting fluorescent protein. (C) The FRET efficiency depends on the angle between the transition moments of the donor and acceptor fluorophores, ranging from 0% in the vertical position to 100% in the parallel position. (D) Schematic representation of intermolecular and intramolecular FRET biosensors. In the intermolecular FRET biosensor system, the donor (Do) and acceptor (Ac) fluorophores are connected to sensor (S) and ligand (L) domains or vice versa. The sensor domain changes its conformation by sensing the signal and binds to the ligand domain, which brings the donor in close proximity to the acceptor and evokes FRET. In the intramolecular FRET biosensor system, all components are in a single molecule.

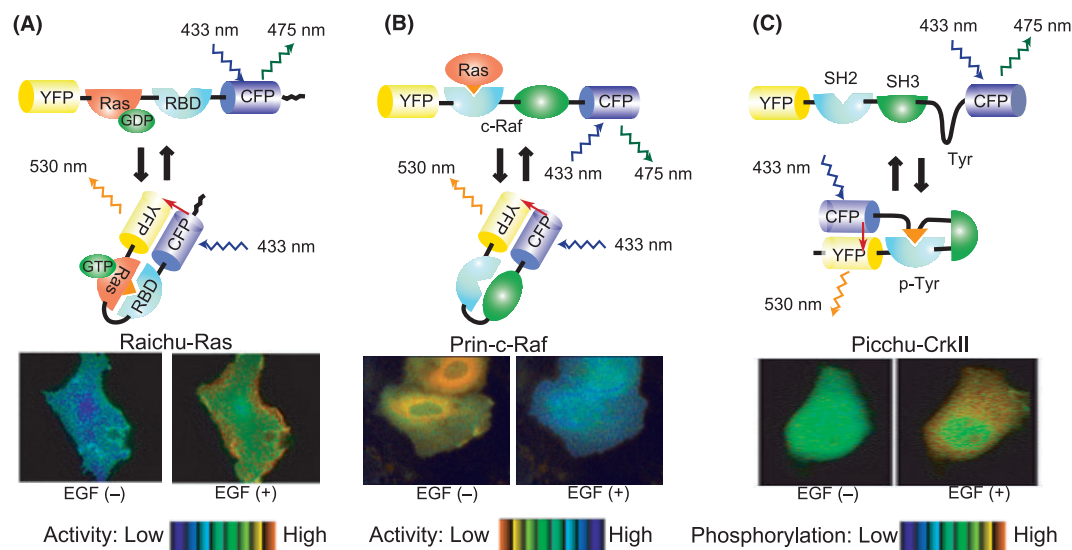


Fig. 2. Intramolecular FRET biosensors based on oncogene products. (A) The Raichu-Ras biosensor uses Ras and the Ras-binding domain of Raf (RBD) as the sensor and ligand domains, respectively. After growth factor stimulation of the cells, activated guanine nucleotide exchange factor promotes GDP exchange with GTP, which causes Ras binding to RBD. Raichu-Ras-expressing cells were illuminated at a wavelength of 433 nm and fluorescence from cyan-emitting fluorescent protein (CFP; 475 nm) and yellow-emitting fluorescent protein (YFP; 530 nm) was quantified. This fluorescence from YFP is often called sensitized FRET. The YFP/CFP value reflects FRET efficiency. (B) Prin-c-Raf monitors the conformational change of c-Raf induced by Ras binding. c-Raf adopts open active and closed inactive conformations as is the case with many serine-threonine kinases. Thus, FRET efficiency is inversely correlated with c-Raf activity in this FRET biosensor design. (C) Picchu-CrkII also monitors the conformational change of the CrkII oncogene product. CrkII is a substrate of epidermal growth factor (EGF) receptor; therefore, CrkII phosphorylation reflects EGF receptor activity.

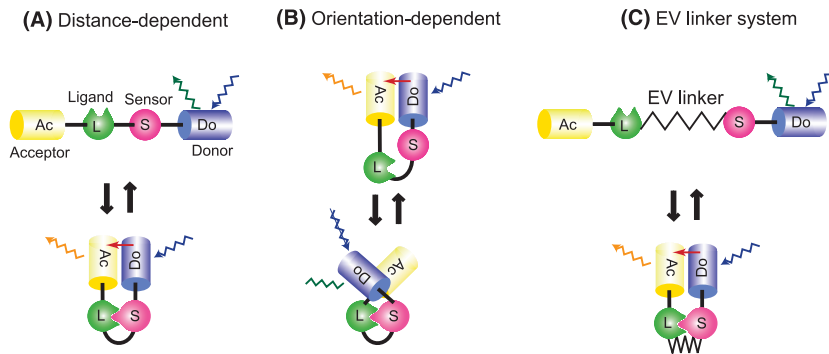
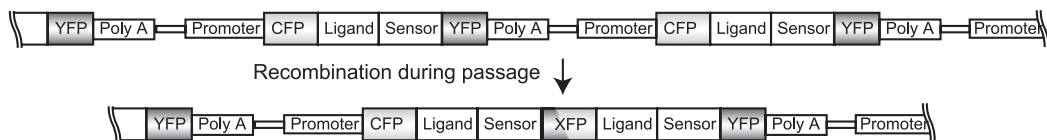
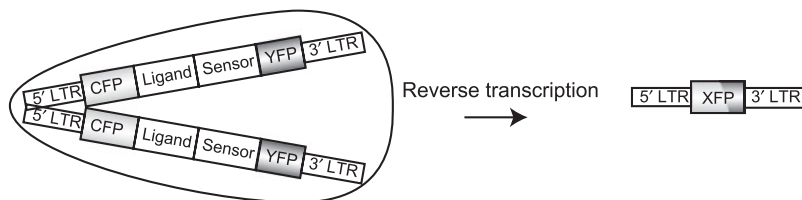


Fig. 3. Mode of action of intramolecular FRET biosensors. (A) Distance-dependent FRET biosensor. The conformational change of the sensor domain (S) causes binding to the ligand domain (L). The FRET efficiency is primarily determined by the distance between donor (Do) and acceptor (Ac) fluorophores. (B) Orientation-dependent FRET biosensor. The FRET efficiency is primarily determined by the angle between the transition dipoles of donor and acceptor fluorophores. (C) EV linker is a flexible 116 amino-acid linker that connects the sensor and ligand domains. Because of its length, the basal FRET caused by the dimerization of donor and acceptor is almost completely abolished, rendering this biosensor the distance-dependent type.

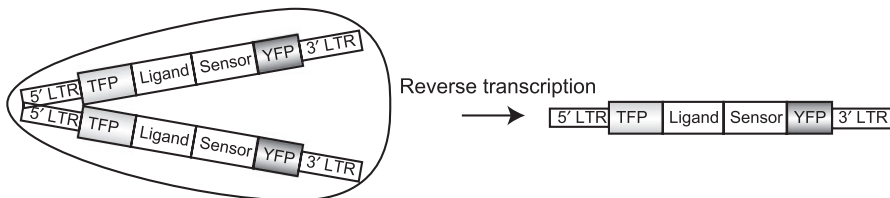
(A) Transfection of the linearized CFP/YFP-type FRET biosensor gene



(B) Retrovirus-mediated transfer of the CFP/YFP-type FRET biosensor gene



(C) Retrovirus-mediated transfer of the TFP/YFP-type FRET biosensor gene



(D) Transposon-mediated transfer of the CFP/YFP-type FRET biosensor gene

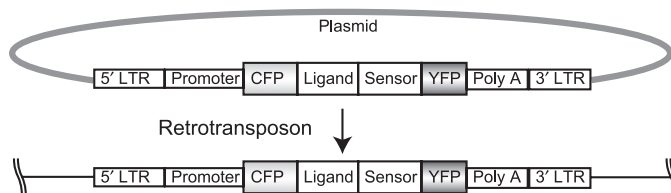


Fig. 4. Establishment of cell lines stably expressing FRET biosensors. (A) Transfection of the linearized CFP/YFP-type FRET biosensor gene. After transfection or electroporation, the linearized expression plasmids of the FRET biosensor are inserted into the genome as a tandem concatamer. Repeated passage of the cells expressing the cyan-emitting fluorescent protein/yellow-emitting fluorescent protein (CFP/YFP)-type FRET biosensor often results in recombination between the CFP gene and the YFP gene. The recombined protein is denoted as XFP. (B) Retrovirus-mediated transfer of the CFP/YFP-type FRET biosensor gene. A retrovirus carries two copies of the genome and uses both for the synthesis of cDNA. High sequence homology between the CFP gene and the YFP gene frequently causes recombination during retrovirus-mediated gene transfer to the host genome. (C) Retrovirus-mediated transfer of the TFP/YFP-type FRET biosensor gene. When the teal fluorescent protein (TFP) gene derived from coral is substituted for CFP derived from jellyfish, the recombination does not take place. (D) Transposon-mediated transfer of the CFP/YFP-type FRET biosensor gene. The piggyBac retrotransposon-mediated gene transfer system can efficiently integrate the FRET biosensor gene without any recombination.

FRET biosensors is laborious work, mostly because the pair of sensor and ligand domains for the monitoring of protein activities and the order of the four components, that is, the acceptor, donor, sensor, and ligand domains, can be optimized only by trial and error. Nevertheless, 15 years after the first report of an intramolecular FRET biosensor,⁽¹¹⁾ many research groups have developed a number of intramolecular FRET biosensors that monitor ion concentrations, sugars, phospholipids, protein kinase activities, small GTPase activities, and so on (<http://www.lif.kyoto-u.ac.jp/labs/fret/e-phogemon/unifret.htm>). As examples, we describe some representative FRET biosensors that monitor the activity of oncogene products (Fig. 2). Rai-chu-Ras, the FRET biosensor for Ras, shows high Ras activity at lamellipodia induced by epidermal growth factor (EGF) stimulation (Fig. 2A). Prin-c-Raf, the biosensor for c-Raf, shows the recruitment of c-Raf to the plasma membrane and concomitant activation of c-Raf on EGF stimulation. Note that FRET efficiency was inversely correlated with Raf activity in this intramolecular FRET biosensor (Fig. 2B). Picchu-CrkII shows that phosphorylation of CrkII diffusely increased in the cytoplasm on EGF stimulation (Fig. 2C). Recently, a modified version of Picchu-CrkII was used to detect Abl tyrosine kinase activity in CML patients,⁽¹²⁾ paving the way to the first clinical use of the genetically encoded FRET biosensors.

Development of an Optimized Backbone for FRET Biosensors

As already described, the developers of FRET biosensors had to expend much effort to design and optimize various parts of the biosensors. To accelerate the development of FRET biosensors, we recently set an optimized backbone for the genetically encoded intramolecular FRET biosensor.⁽¹³⁾ First, we determined the optimal donor and acceptor fluorescent proteins. Several research groups have optimized fluorescent proteins for FRET biosensors.^(14–16) Because most recent biosensors use a cyan-emitting mutant (CFP) as the donor and a yellow-emitting mutant of GFP (YFP) as the acceptor, we concentrated on the comparison among CFP-like and YFP-like fluorescent proteins such as CyPet, Ypet,⁽¹⁶⁾ Venus,⁽⁹⁾

Turquoise,⁽¹⁷⁾ and teal fluorescent protein (TFP).⁽¹⁸⁾ Among the tested fluorescence proteins, enhanced CFP and Turquoise served as the best donor fluorophores with the use of YPet as the acceptor fluorophore.

Other than the FRET pair, the FRET efficiency of FRET biosensors is determined by the distance and the relative orientation of the donor and acceptor fluorophores (Fig. 3). In the distance-dependent mode (Fig. 3A), FRET will increase when the sensor domain binds to the ligand domain. In the orientation-dependent mode, FRET is high before the sensor domain binding to the ligand domain (Fig. 3B). Fluorescent proteins derived from GFP tend to dimerize each other and such dimerization is known to enhance FRET efficiency.⁽¹⁹⁾ Thus, in contrast to the distance-dependent mode, on the binding of the sensor domain to the ligand domain, the FRET efficiency will decrease due to the rotation of the FRET pair (Fig. 3B). Many FRET biosensors show the distance-dependent mode and a smaller number of the FRET biosensors show the orientation-dependent mode.^(20,21) Unfortunately, in the absence of the 3-D structures of the FRET biosensors, it is difficult to predict which of these two modes will be dominant in each biosensor design. We overcame this problem by the use of a very long linker, named the EV linker, that connects the ligand domain to the sensor domain.⁽¹³⁾ The EV linker consists of 116 amino acids and thus abolishes the dimerization of the FRET pair, rendering the FRET efficiency mostly dependent on distance. With this system, the gains of FRET biosensors of ERK, PKA, Ras, and Rac1 were markedly increased and new biosensors for S6K and RSK were made without many optimization steps. This EV linker system will accelerate the development of FRET biosensors for kinases and small GTPases, which will help to decipher the role of signaling molecules in living cells.

Techniques to Establish Cell Lines Stably Expressing FRET Biosensors

An unspoken flaw of FRET biosensors is the difficulty of establishing cell lines that stably express FRET biosensors comprised of CFP and YFP. There has been a study using a cell line stably expressing such a biosensor,⁽²²⁾ but most

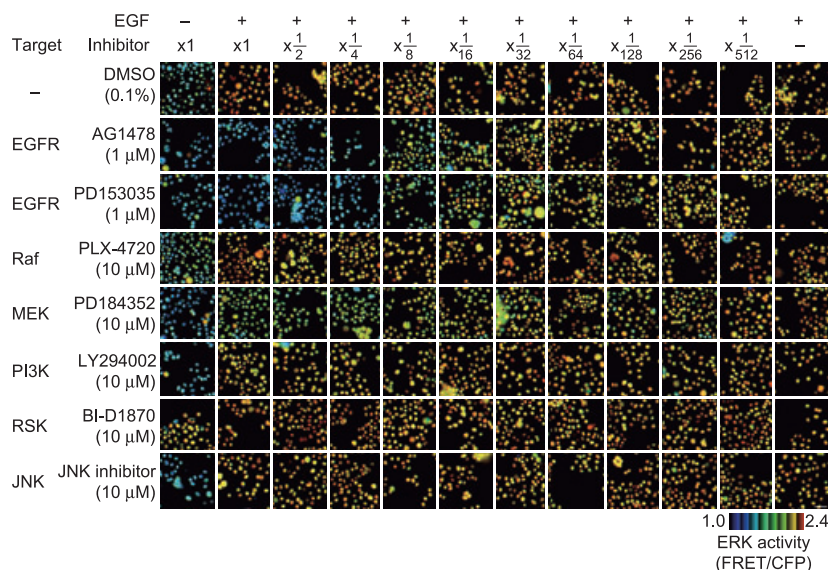


Fig. 5. Förster (or fluorescence) resonance energy transfer imaging-based assay of ERK sensitivity to kinase inhibitors. HeLa cells stably expressing a FRET biosensor for ERK, EKAREV-nuc, were pretreated with (+) or without (–) 25 ng/mL epidermal growth factor (EGF) and kinase inhibitors.⁽¹³⁾ Kinase inhibitors and their target kinases were: AG1478 (EGF receptor [EGFR] inhibitor); PD153035 (EGFR inhibitor); PLX-4720 (Raf inhibitor); PD184352 (MEK inhibitor); LY294002 (PI3K inhibitor); BI-D1870 (RSK inhibitor); and JNK inhibitor VIII (JNK inhibitor). CFP, cyan-emitting fluorescent protein.

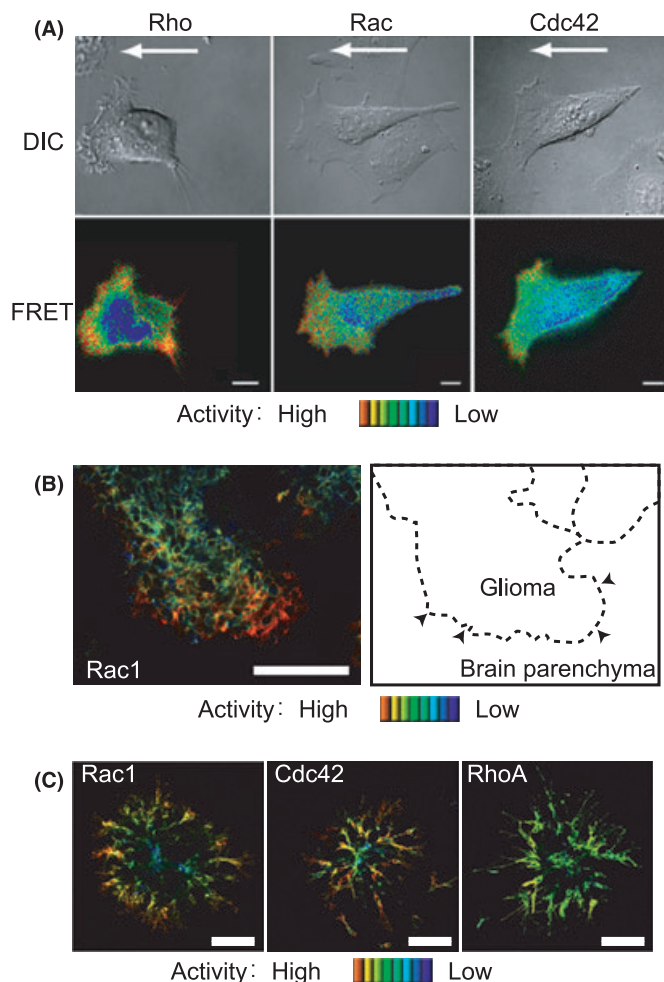


Fig. 6. Spatiotemporal regulation of Rho-family GTPases within cells, in 3-D gels, and in tumor tissues. (A) MDCK cells transiently expressing a FRET biosensor for RhoA, Rac1, or Cdc42 were time-lapse imaged. Differential interference contrast (DIC) and FRET images are shown. Note that RhoA is active both at the lamellipodia at the front and uropods at the end. In contrast, Rac1 and Cdc42 are active specifically at lamellipodia. Arrows indicate the direction of cell migration. Scale bar = 10 μ m. (B) C6 glioma cells stably expressing a FRET biosensor for Rac1 were inoculated into a rat brain. Ten days after inoculation, the brain slice culture was prepared and observed with a confocal microscope. Glioma cells invading into the brain parenchyma showed higher Rac1 activity than those in the center of the tumor mass. Arrowheads indicate the invasion front. Scale bar = 100 μ m. (C) C6 glioma cells stably expressing FRET biosensors for Rho-family GTPases were embedded in Matrigel and grown for 2 days.⁽³¹⁾ Glioma cells invading into the gels show higher Rac1 and Cdc42 activities than those following the leading cells. RhoA activity was almost homogeneous among the glioma cells. Scale bar = 100 μ m.

studies with FRET biosensors have used transient transfection for the expression. Transfection/electroporation of linearized plasmid DNAs and retroviral induction are the two major methods for the establishment of cell lines stably expressing exogenous proteins. In our experiments, transfection of a linearized expression plasmid of a FRET biosensor produced cells expressing a functional FRET biosensor for a short period; however, during repeated cloning steps, cells almost always lost the expression of CFP, YFP, or both (Fig. 4A). With retrovirus-mediated gene transfer, most infected cells expressed either CFP or YFP (Fig. 4B). Retroviruses carry two copies of the RNA genome. The RNA genome is often fragmented in the virus particle; therefore, the reverse transcriptase uses the

two copies of RNA genome to replicate the full genome without mistake.⁽²³⁾ This means that the reverse transcriptase on one copy of the genome RNA can jump on to the other copy of the genome RNA easily. Thus, we speculated that, due to the high sequence homology between CFP and YFP, the reverse transcriptase may jump from the CFP-coding region to the YFP-coding region or vice versa. Under this assumption, we changed the donor fluorophore from CFP to TFP, a fluorescent protein derived from coral.⁽²⁴⁾ As we anticipated, FRET biosensors carrying TFP and YFP as a FRET pair were readily expressed by retrovirus-mediated gene transfer, without any recombination (Fig. 4C).⁽²⁵⁾

When the donor fluorophore was changed from CFP to TFP, however, the gain of FRET biosensors was almost always decreased.⁽¹³⁾ We recently found that piggyBac transposon-mediated gene transfer can be used to efficiently establish cell lines stably expressing FRET biosensors comprised of both CFP and YFP.⁽²⁵⁾ These cell lines are very useful for the assessment of the effect of anticancer drugs on the signaling molecules (Fig. 4D). We adduce a HeLa cell line stably expressing a FRET biosensor for ERK as an example (Fig. 5). ERK activity was chosen as the readout of the canonical oncogene signal transduction cascade comprised of EGFR, Ras, and Raf. There are several merits for using this cell line for the screening of anticancer drugs. First, the cell-based assay guarantees fairly good drug-delivery into the cells for the hit-compounds. Second, multiple potential drug targets in the oncogene signal transduction pathway can be screened simultaneously. Third, the time-course of the effect of drugs can also be acquired in a single experiment. Fourth, the number of cells may be scaled down to as few as 100 cells. Finally, up to three different FRET biosensors can be used in a single experiment. This is because FRET biosensors localized to the cytoplasm, nucleus, and plasma membrane can be distinguished by an image-processing program.

Monitoring Signaling Activities in 3-D Culture and Living Tissues

Next we adduce Rho-family GTPases as examples to indicate the merit of FRET biosensors. Rho-family GTPases play a central role in the regulation of invasion by cytoskeletal reorganization. The coordinated activation and/or antagonistic action of Rho-family GTPases determine the invasion morphologies of cancer cells.⁽²⁶⁾ Three major members of Rho-family GTPases, RhoA, Rac1, and Cdc42, are associated with the three characteristic subcellular architectures, stress fiber, lamellipodia, and filopodia.⁽²⁷⁾ In agreement with these proposed roles, FRET biosensors have visualized high RhoA activity at the tail and front, and high Rac1 and Cdc42 activities at the front of migrating cells (Fig. 6A). Thus, FRET biosensors of Rho-family GTPases have been proven to be powerful tools for revealing subcellular activity maps.

How, then, are Rho-family GTPases regulated at the level of tissues? Two-photon intravital microscopy has revealed that cancer cells show diverse invasion morphologies in tissues.^(28,29) It has been proposed that the balance between RhoA and Rac1 may determine the mode of cancer cell invasion.⁽³⁰⁾ Using the newly developed cell lines stably expressing FRET biosensors for Rho-family GTPases, we have shown the spatial activity maps of Rho-family GTPases with glioma cells invading into brain parenchyma.⁽³¹⁾ We found that Rac1 activity was high in the cells invading into the brain parenchyma at the front of glioma cells (Fig. 6B). Two scenarios could be sketched to explain this observation. Glioma cells may be activated at the front of the invasion, probably by certain growth factors. Alternatively, glioma cells showing higher Rac1 activity may guide the other cells showing lower Rac1 activity. To

determine which scenario applies, glioma cells stably expressing FRET biosensors for Rac1, Cdc42, or RhoA were grown in 3-D matrigel, wherein the concentration of growth factors should be homogenous (Fig. 6C). We found that cells with high Rac1 activity guided the other glioma cells in 3-D Matrigel. Similar data were obtained for Cdc42. RhoA did not show such a difference and served as a good control.

Future Perspectives of FRET Biosensors in Cancer Research

Now that an optimized backbone for FRET biosensors has been established, a number of FRET biosensors for kinases and small GTPases should be available in the near future. It would be very exciting to see how signaling pathways other than the EGFR-Ras-ERK pathway are spatiotemporally controlled in cancer cells and tissues. We have already reported on the S6K biosensor, which visualizes the activity of the mTORC1 pathway.⁽¹³⁾ Biosensors for the Cdk family⁽³²⁾ or others will also shed new light on the spatial control of signaling cascades related to oncogenesis. Use of cell lines stably expressing FRET biosensors could potentially replace SDS-PAGE and immunoblotting analysis. The activity changes of kinases, small GTPases, and/or phosphoinositides in cancer cells will be visualized during the initial transformation, inva-

sion, and metastasis of cancer cells. The screening and validation of anticancer drugs can be accelerated with the cell lines described here and being developed. Moreover, the transposon-mediated gene transfer technique should also be applicable for the generation of transgenic mice. Such mice will provide ideal animals to examine the pharmacodynamics of anticancer drugs in living animals. Thus, stable expression of FRET biosensors will accelerate current trends in cancer research, that is, from cells on a plastic dish to 3-D and/or live tissues, and from biochemistry to live imaging.

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Disclosure Statement

The authors have no conflict of interest.

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