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Review

Fluorescence bioimaging of intracellular signaling and its clinical application

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ABSTRACT

Background: Fluorescent proteins have continued to shed light on cell biology since the cDNA of wild type green fluorescent protein was first isolated. Nowadays, these remarkable proteins are useful tools, not only in basic research, but also in clinical medicine.
Highlight: By taking advantage of fluorescent protein-based technologies, we identified a signaling network critical for influenza virus internalization and infection. In addition, we developed a highly sensitive biosensor for monitoring kinase activity that utilizes energy transfer between fluorescent proteins. This has led to a high-performance clinical test that enables the prediction of future therapeutic responses and the risk of acquired drug resistance for each individual patient before beginning molecular target therapy.
Conclusion: Technologies that utilize fluorescent proteins, such as the biosensor presented here, should find increasing applications in clinical medicine.

Contents

1. Introduction

Green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea victoria* by Dr. Osamu Shimomura [1], has continued to shed light on cell biology since its cDNA was isolated [2]. Because GFP can generate its intrinsic chromophore without cofactors or enzymatic components [3], it can be used as a fluorescent marker inside cells that have been transfected with a gene

Abbreviations: BAPTA-AM, acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BiFC, bimolecular fluorescence complementation; CML, chronic myeloid leukemia; CFP, cyan-emitting mutant of green fluorescent protein; FRET, Förster resonance energy transfer; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; IM, imatinib; IP_3, inositol 1,4,5-triphosphate; MDC, monodansylcadaverine; PI3K, phosphoinositide 3-kinase; PI(4)P, phosphatidylinositol-4-phosphate; PI(4,5)P2, , phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; RalGDS, Ral guanine nucleotide dissociation stimulator; SH, src homology; YFP, yellow-emitting mutant of green fluorescent proteins

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encoding GFP. In addition, the development of color variants of GFP and isolation of fluorescent proteins from organisms other than jellyfish [4] together make it possible to "illuminate" living cells using a palette of organelle markers tagged with a variety of fluorescent proteins. Not only the locations of proteins, but also their interactions or activations can be monitored by using the principles of reconstitution of protein fragments and/or a physicochemical energy transfer phenomenon.

Fluorescent proteins are useful tools not only for basic science but also for clinical research. We have developed a biosensor that can be used clinical tests [5]. The biosensor monitors protein tyrosine kinase activity in living, patient-derived tumor cells and allows the evaluation of kinase inhibitor efficacy. With this biosensor, we can now detect small population of drug resistant cells and predict future drug responses of patients, which may lead to a new era in clinical cancer research. Here, we would like to review examples of both basic and clinical research in which fluorescent proteins were used to provide solutions for difficult challenges.

2. Host cell signaling that is critical for influenza virus entry and infection

2.1. Regulation of endocytosis and virus particle internalization by Ras-PI3K signaling

Ras, a small G protein, acts as a molecular switch in intracellular signal transduction pathways and regulates a variety of pathophysiological functions (Fig. 1a). The inactive form of Ras binds to GDP, and is activated by replacement of bound GDP with GTP, which is mediated by guanine nucleotide exchange factors (GEFs) [6]. Activated Ras reverts to the inactive form with the hydrolysis of GTP to GDP, which is accelerated by GTPase activating proteins (GAPs). Only the active form of Ras can bind to effector molecules and stimulate downstream signaling. More than 10 effector molecules have been identified to date, including the lipid kinase phosphoinositide 3-kinase (PI3K), the serine/threonine kinase Raf, and Ral guanine nucleotide dissociation stimulator (RalGDS). This diversity in effector molecules is the reason Ras is called a molecular switch; Ras has no enzymatic activity other than GTP hydrolysis, but utilizes multiple effector molecules to participate in a variety of cellular functions, including proliferation, differentiation, motility, cell death and survival, vesicular trafficking, as well as long-term potentiation. The function and activity of Ras are regulated not only by the balance between activities of GEFs and GAPs, but also by spatiotemporal regulation of effector recruitment.

To visualize where and when Ras binds to specific effector molecules, we utilized a bimolecular fluorescence complementation (BiFC) technique (Fig. 1b). A range of proteins can be divided into two fragments with no function, and a functionally active complex can be reconstituted when they are brought in proximity to each other. Fusing the fragments to partners that interact with each other enhances the efficacy of their assembly. In the case of fluorescent proteins, an interaction between proteins fused to non-fluorescent fragments of a fluorescent protein facilitates the reconstitution of the fragments, leading to functioning fluorescence [7]. In Fig. 1b, fluorescence emission can be observed in a manner dependent on an interaction between Ras and an effector molecule. The plasmids encoding these proteins were introduced into cells, and the change of fluorescence intensity originating from the unified fragments of the expressed proteins was monitored using fluorescence microscopy.

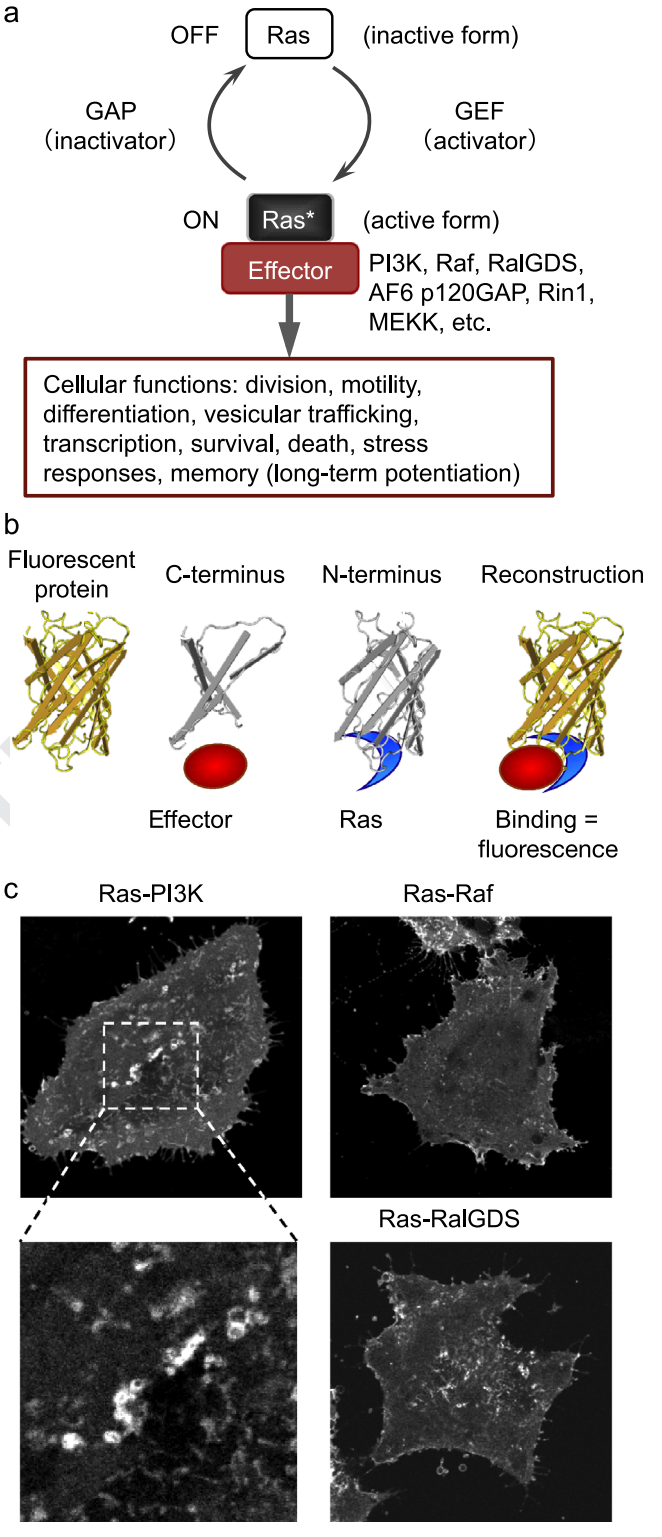

Fig. 1. Ras-PI3K complex is localized in endosomes. (a) Regulation of the Ras family of small G proteins. GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein. (b) Schematic representation of bimolecular fluorescence complementation (BiFC). The N-terminal and C-terminal fragments of fluorescent proteins emit no fluorescence, however reconstitution of the whole protein from the fragments results in fluorescence emission. Tagging of the fragments with interacting peptides can facilitate the assembly of the fluorescent protein constituents. (c) Cos-1 cells were transfected with expression vectors for the purpose of monitoring Ras and the effector complex as indicated, and then imaged on a confocal microscope. The left lower panel is a magnified image of the inset in the left upper panel.

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When Cos-1 cells were transfected with BiFC plasmids and observed under confocal microscopy, most Ras-effector complexes were localized at the plasma membrane (Fig. 1c). This localization might be accounted for by membrane recruitment of effector molecules by Ras, which was reported to reside at the inner leaflet of the plasma membrane (attached through the farnesylation moiety at its C-terminus). In addition to the fluorescence at the plasma membrane, intracellular vesicular structures were visualized by the fluorescent Ras-PI3K complex (Fig. 1c). Such vesicular structures were formed at the peripheral region of the plasma membrane and moved to the central region in the presence of EGF, indicating that these vesicles are endosomes. By immunofluorescence staining along with the BiFC assay, it was also clearly revealed that the Ras-PI3K complexes were indeed localized in early endosomes, and PI3K molecules recruited by Ras in the endosome are indeed activated [8].

By utilizing mouse embryonic fibroblasts deficient in a class of PI3K and reconstitution experiments with a PI3K mutant that cannot bind to Ras, it was shown that Ras-PI3K signaling from endosomes is indispensable for the regulation of clathrin-independent endocytosis, endosomal maturation, and intracellular transport of influenza viruses. These results demonstrate that the Ras-PI3K signaling axis acts as a host-oriented mechanism for viral internalization. Given that virus incorporation is a process conserved among virus subtypes and species, this signaling pathway may provide a target for potent, well-tolerated prophylactics and therapeutics against a broad range of viruses [9].

2.2. Host cell signaling that regulates influenza virus particle internalization

Next, we investigated how Ras-PI3K signaling is activated by influenza viruses. It has been reported that Ras is activated by calcium signaling and tyrosine phosphorylation [6]; therefore, we tested whether these signaling pathways are activated by influenza viruses. Whereas the status of intracellular tyrosine phosphorylation was not altered by infection, as analyzed by western blotting, Cameleon, a calcium biosensor based on the principle of Förster resonance energy transfer (FRET, see also Fig. 3b for FRET) successfully demonstrated that transient, repeated increases in calcium concentration were induced after influenza virus infection (Fig. 2a). In addition, pretreatment with the calcium chelator acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) completely inhibited virus-dependent activation of Ras, indicating that Ras activation by influenza viruses is mediated by intracellular calcium signaling.

To evaluate the significance of intracellular calcium in influenza virus infection, we utilized a plaque assay and found that treatment with BAPTA-AM dramatically suppressed viral replication. It was to our surprise that the efficacy of BAPTA-AM was much greater than that of oseltamivir, one of the most popular anti-influenza drugs, suggesting that intracellular calcium is critical for influenza virus infection. It should also be considered that the inhibitory effect of BAPTA-AM is much higher than that of the PI3K inhibitor LY294002, although we identified calcium signaling as the upstream regulator of Ras-PI3K signaling. This result indicates that intracellular calcium plays a pivotal role in influenza virus infection not only through the regulation of clathrin-independent endocytosis, but also through the regulation of other signaling pathways.

In order to investigate other pathways besides clathrin-independent endocytosis, we revisited the endocytic pathways that are utilized in influenza virus entry. Classically, it is well established that influenza viruses are internalized via clathrin-mediated endocytosis [10,11]; however, it has also been reported that inhibition of this pathway did not prevent infection [11–13]. Our group and others recently reported that influenza viruses are also internalized via clathrin-independent endocytosis [13,14]. Therefore, we hypothesized that calcium regulates not only clathrin-independent endocytosis, but also clathrin-mediated endocytosis, and tested the hypothesis using multiple inhibitors and combinations of them. Inhibition of clathrin-independent endocytosis by the PI3K inhibitor LY294002 partially suppressed infection by the virus, and treatment with monodansylcadaverine (MDC), an inhibitor for clathrin-mediated endocytosis, displayed no effect on the infection, as reported previously [13]. Therefore, upon inhibition of one pathway, influenza viruses can still be internalized through another pathway. Simultaneous treatment of MDC and LY294002 dramatically inhibited virus infection, and the effect is comparable to that of the calcium chelator BAPTA-AM. Therefore, the data suggested that calcium regulates both clathrin-mediated and -independent endocytosis.

To confirm that the above blockage of infection is indeed due to inhibition of viral particle internalization, cells expressing the late endosome marker Rab7 were challenged with influenza viruses, and then fixed and subjected to immunofluorescence using an antibody against NP, one of the virus components. In control cells, internalized particles were colocalized with late endosomes (Fig. 2b); however, such colocalization was dramatically decreased under calcium chelation or inhibition of endocytosis. In summary, calcium signaling mediates influenza virus entry through the regulation of multiple endocytic pathways.

In addition to the core role of calcium signaling in influenza virus internalization, we also identified a signaling network through which viral particles can be incorporated into cells (Fig. 2c). First, influenza viruses adsorb to sialylated cell surface proteins, which in turn activate calcium channels through unknown mechanisms. An increase in intracellular calcium activates the RhoA-ROCK-PIP5K pathway. Production of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] from phosphatidylinositol-4-phosphate [PI(4)P] results in the activation of Epsin1 and subsequent clathrin-mediated endocytosis, through which influenza viruses are incorporated into cells. PI(4,5)P_2 is also transformed into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol by phospholipase C (PLC). IP_3 then induces calcium release from the ER and activates the positive feedback loop. Calcium and diacylglycerol activate CalDAG-GEF, Ras, PI3K, and Rac1, which accelerates clathrin-independent endocytosis. By activating this signaling circuit, influenza viruses might be efficiently internalized into cells via both clathrin-independent and -mediated endocytosis [15]. Following this discovery, we are now engaged in trying to identify the molecular mechanism through which influenza viruses activate cell surface calcium channels.

3. Clinical application of bioimaging

3.1. Development of a FRET-based biosensor for the measurement of BCR-ABL activity

Our laboratory is currently involved in expanding the application of bioimaging techniques into the clinical arena with chronic myeloid leukemia (CML) as a model disease. CML is a myeloproliferative disease characterized by the emergence of Philadelphia chromosome, which arises from a reciprocal translocation between chromosomes 9 and 22. The genes located at the break point, *Bcr* and *Abl*, form a new fusion gene, *Bcr-Abl*, and its subsequent transcript encodes the chimeric tyrosine kinase BCR-ABL. The constitutive activity of this kinase induces sustained phosphorylation of substrates, resulting in malignant transformation. Imatinib mesylate, a specific inhibitor of the Abl kinase, can ablate

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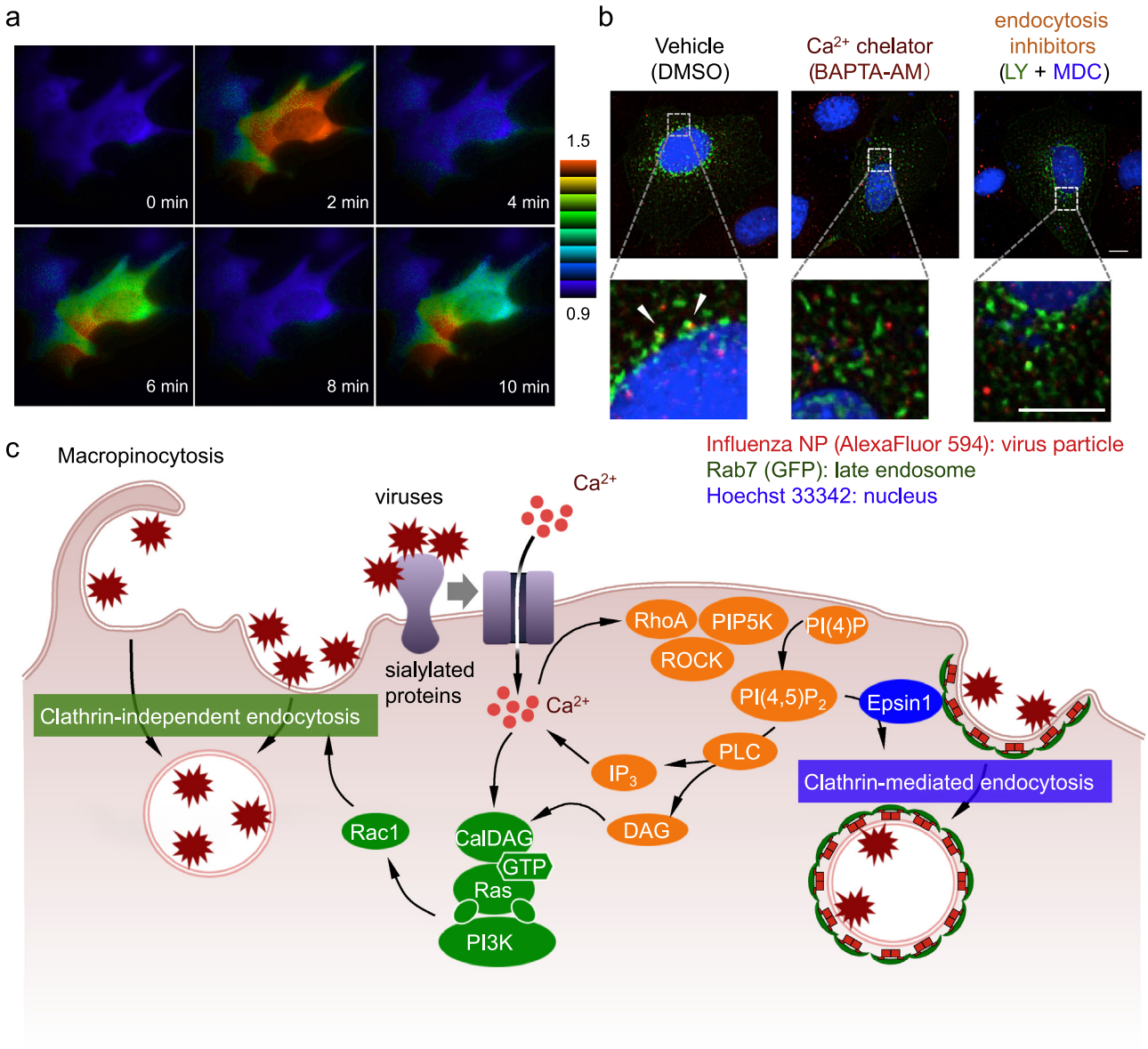

Fig. 2. Intracellular calcium is a key regulator for influenza virus internalization and infection. (a) Mouse embryonic fibroblasts transfected with an expression vector for the FRET-based Ca^{2+} sensor YC3.60 were monitored by dual-emission fluorescence microscopy. Cells were exposed to PR8 or mock infection at time 0. FRET/CFP ratio images were generated to represent FRET efficiency, and the upper and lower limits of the ratio range are indicated. (b) MDCK cells transfected with an expression vector for GFP-Rab7 were incubated in the absence or presence of BAPTA-AM or LY294002 and MDC (LY + MDC) for 30 min before infection with PR8 for 1 h at 37 °C. DMSO was used as a negative control. The cells were then fixed and stained for immunofluorescence using an anti-NP antibody. (c) Model for the intracellular signaling circuit that underlies the regulation of influenza virus internalization via clathrin-mediated and -independent endocytosis.

this linkage, and is therefore expected to be an effective therapeutic opposing the progression of the cancer.

In fact, this reagent has succeeded in transforming CML into a disease controllable by oral medication. In the first clinical trial, in which overall survival of patients treated with imatinib was compared with the survival of those treated with conventional therapies, imatinib achieved a dramatic outcome far superior to those of any previously available therapies, including chemotherapy, bone marrow transplantation, and interferon [16,17]. However, because these patients are never completely cured, they are required to continue undergoing drug treatments. A more serious concern is that drug resistance often develops as the percentage of cells able to survive and reproduce in the presence of the drug increases in the population (Fig. 3a). At the initial diagnosis, most CML cells are imatinib sensitive and can be eliminated by the treatment. However, drug-resistant cells become proliferative during the treatment and eventually replace the major population of CML.

The development of technology to detect a small number of resistant cells before treatment is urgently desired. To accomplish this goal, we have introduced bioimaging methods, fluorescent proteins, and FRET, into this clinical setting. FRET is a radiationless excitation energy transfer from a donor chromophore to an acceptor chromophore [18,19], which can be observed only when the donor and acceptor are very close to each other. In the case of GFP-based FRET, cyan- and yellow-emitting mutants of GFP (CFP and YFP) have often been used as donor and acceptor chromophores, respectively. When CFP alone is excited by 440-nm light, cyan fluorescence (emission wavelength of ca. 480 nm) emanates from CFP. In contrast, when YFP exists near enough to CFP (normally less than 10 nm), a transfer of excitation energy from CFP to YFP occurs, thereby resulting in YFP emission (Fig. 3b). To detect BCR-ABL activity by FRET, one of its major substrates, CrkL, was

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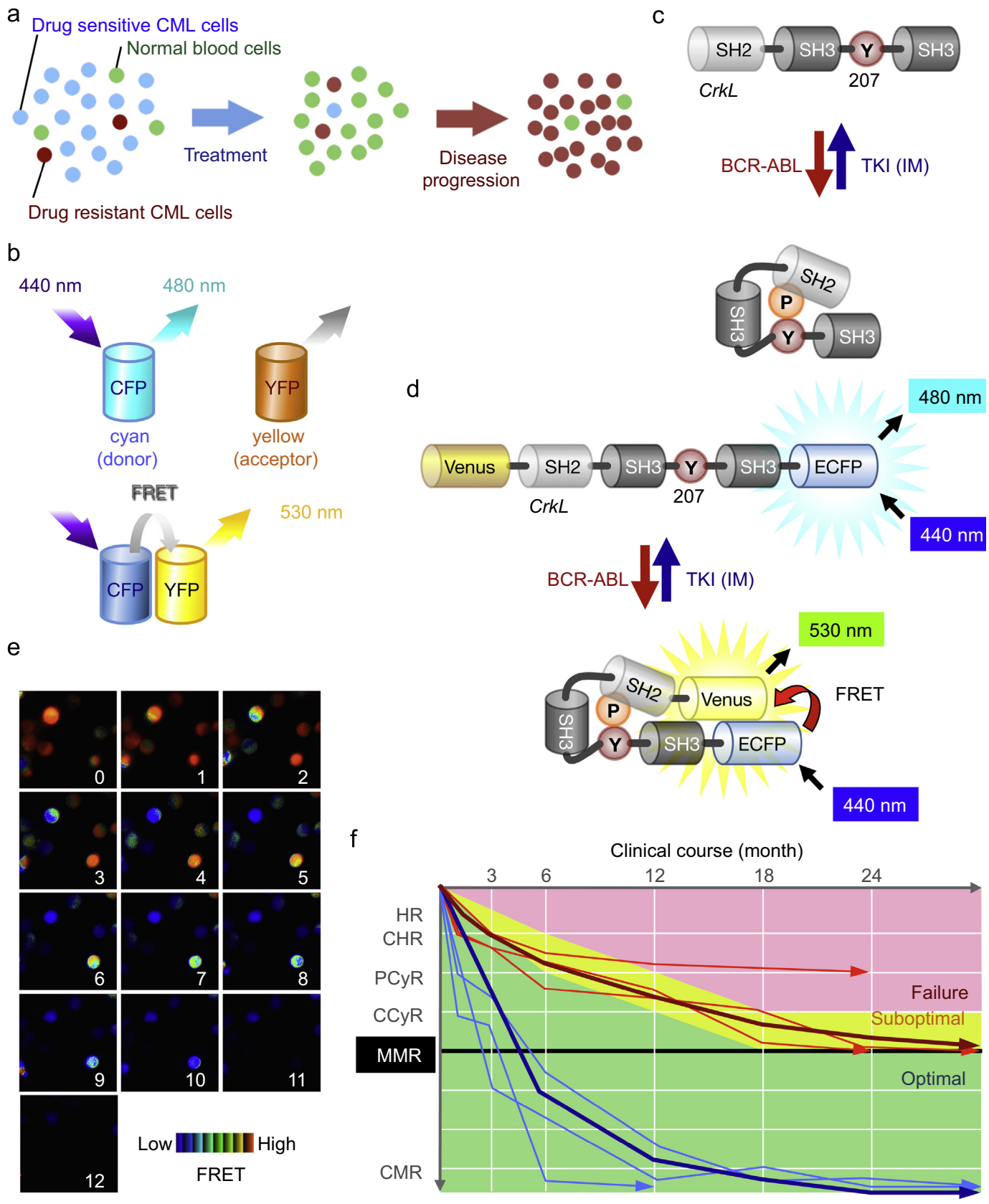

Fig. 3. Clinical application of FRET-based biosensors. (a) Model for the repopulation of drug resistant CML cells. (b) The principle of Förster resonance energy transfer. (c) The structure of the adaptor protein CrkL and its conformational change induced by tyrosine phosphorylation at tyrosine (Y) 207. SH, src homology; TKI, tyrosine kinase inhibitor; IM, imatinib. (d) Schematic representation of the phosphorylation indicator of CrkL *en* substrate, which was developed for accurate measurement of BCR-ABL activity. Venus and ECFP are brighter mutants of YFP and CFP, respectively. (e) K562 cells expressing Pickles were subjected to time-lapse fluorescence microscopy. The emission ratio and the intensity of ECFP were used to generate the reconstituted images using intensity-modulated display (IMD) mode. Photographs from the indicated time points after IM treatment (hour) are shown. (f) Clinical course of patients who were diagnosed as IM-sensitive (blue) and IM-resistant (red) by FRET-based clinical test. Fine lines indicate the clinical course of each patient (three from each group), while bold lines represent the mean of ten cases. Based on the relationship between time after treatment and disease status, patient responsiveness is categorized into three groups: optimal response (Optimal), suboptimal response (Suboptimal), and failure, by European Leukemia Net. HR, hematological response; CHR, complete hematological response; PCyR, partial cytological response; CCyR, complete cytological response; MMR, major molecular response; CMR, complete molecular response.

recruited. Upon phosphorylation of tyrosine 207 on CrkL by BCR-ABL, the intramolecular binding between the src homology 2 (SH2) domain and the phosphorylated tyrosine induces a conformational change in CrkL (Fig. 3c). Treatment with tyrosine kinase inhibitors, in contrast, revert it to the open conformation. Therefore, by adding variants of YFP and CFP (Venus and ECFP, respectively) at the N- and C-termini of CrkL, respectively, a FRET-based biosensor was designed. With this biosensor, BCR-ABL activity can be monitored by an increase in FRET efficiency (Fig. 3d). This molecule is named Pickles, after phosphorylation indicator of CrkL *en* substrate.

The prototype biosensor showed only a marginal difference in FRET efficiencies when BCR-ABL was present versus absent. However, by introducing several improvements, we obtained a biosensor that displayed a marked increase in FRET efficiency upon CrkL phosphorylation. CML cells were transfected with an expression plasmid for this biosensor and observed with a fluorescence imaging workstation, followed by calculation of FRET efficiency based on the acquired images. As shown in Fig. 3e, the initially high BCR-ABL activity in CML cells (indicated in red) gradually decreased (turned blue), in response to imatinib treatment. Therefore, BCR-ABL activity can be measured at a single cell level with this biosensor.

In addition to the single cell level evaluation, recently accumulated evidence obtained from our studies indicates that this system can also be a valuable tool for the prediction of drug responses in patients. During five clinical trials, in which Pickles was used to predict future drug responses before treatment for more than 130 patients (Table 1), the predictions correlated very well with the clinical outcomes (concordance rate $>80\%$). Typical examples are shown in Fig. 3f. A criterion regarding preferable therapeutic responses has been defined by the European Leukemia Net, in which patients' drug responses were classified into three categories: optimal response, suboptimal response, and failure, based on treatment duration and disease status, including the level of BCR-ABL mRNA. All of the patients diagnosed as imatinib sensitive by our method (indicated by blue lines) were quick responders, whereas none of the patients who were diagnosed as imatinib resistant (red lines) displayed such a preferable response. Although a number of assay systems have been developed to evaluate the amount or activity of BCR-ABL, our technology is currently the only method that can predict future drug responses (Table 2).

In addition to these advantages, Pickles can be easily utilized in the evaluation of second-generation drugs, such as nilotinib and dasatinib. These second-generation drugs were originally developed for patients with imatinib resistance due to mutations in the kinase domain of the BCR-ABL fusion gene, but now they are available as first-line therapy, regardless of the presence or absence of mutations. Our method can be easily adapted to assays with any drugs.

Table 1
Clinical trials, in which Pickles has been involved. HUHP, Hokkaido University Hospital; BMC, bone marrow cell; PBMC, peripheral blood mononuclear cell.

Project	Specimen	Number of cases
Independent clinical trial in HUHP	(BMC & PBMC)	21
Multicenter trial A	(BMC)	4
Multicenter trial B	(BMC)	19
Multicenter trial C	(BMC)	69
Multicenter trial D	(PBMC)	17
Total		130

Table 2
Clinical tests used for the assessment of CML disease status. Ability of each test in detecting single drug resistant cells or predicting drug response is indicated by ○, △, or ×. *, tests involved in the guidelines for treatment of CML. FISH, fluorescence in situ hybridization; qPCR, quantitative PCR; pCrkL, phosphorylated CrkL; ELISA, enzyme-linked immunosorbent assay.

	Evaluation of BCR-ABL's	Drug resistance detection by single cell analysis	Drug response prediction
FISH*	Existence	×	×
qPCR*	Expression level	×	×
Direct sequencing*	Mutation	×	△
Pickles	Activity	○	○
Western blotting			
BCR-ABL	Expression level	×	×
pCrkL	Activity	×	×
ELISA			
BCR-ABL	Amount	×	×
pCrkL	Activity	×	×
Flow cytometry	Activity	○	△

3.2. Exploration of the molecular mechanisms of drug resistance

Through multiple clinical trials, the effectiveness of the FRET-based biosensor Pickles in the clinical setting has been demonstrated; however, at the same time, we noticed that a substantial proportion of patients display multidrug resistance. This fact encouraged us to explore the molecular mechanisms of drug resistance by taking advantage of a possible mode of our method, namely live-cell analysis. Ph1-positive leukemia cells expressing Pickles were treated with imatinib, and then FRET-high cells, which are assumed to be resistant to imatinib, were isolated from imatinib sensitive cells by using fluorescence-activated cell sorting. First, we tested whether FRET-high cells possesses stem cell properties, because CML has been demonstrated to be a clonal disease, (originating from a pluripotent hematopoietic stem cell) and it might be a particularly important issue whether leukemia stem cells respond to treatment or not. To do so, we performed a colony formation assay using a methylcellulose gel, and unexpectedly found that colony formation activity of FRET-high cells was not higher, but rather lower than that of FRET-low cells. Therefore, at least under our assay conditions, leukemia stem cells are not enriched in an imatinib resistant cell population.

To elucidate the molecular mechanisms that lead to drug resistance, we compared the gene expression patterns between FRET-high and -low cells by cDNA microarray, and identified large numbers of upregulated and downregulated genes. Through annotation analysis, we identified 24 upregulated signaling pathways in FRET-high cells. We focused on a certain signaling pathway, because inhibitors that can be used clinically are available that will inhibit the late limiting enzyme of the pathway. Cell cycle analysis by flow cytometry revealed that simultaneous treatment with imatinib and the inhibitor resulted in a dramatic alteration of cell cycle progression, whereas each drug has only minimal effects on cell cycle progression at the same dose. Given that the sub-G1 population was significantly increased, imatinib, in the presence of the inhibitor, induced apoptotic cell death in Ph1-positive leukemia cells. Consistently, the inhibitor could prevent, in a dose-dependent manner, the repopulation of imatinib resistant cells that is usually observed during long-term culture in the presence of imatinib. Therefore, these results indicate that the inhibitor can sensitize drug resistant leukemia cells to imatinib, and may provide an effective therapeutic option for patients with imatinib-resistant leukemia.

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4. Conclusions

Currently, fluorescent proteins and related techniques, including BiFC and FRET, are commonly used in a wide range of biological sciences; however, they have been limited to basic research. The studies reviewed here should draw attention to the usefulness of GFP in clinical medicine. In fact, the published paper regarding Pickles was highlighted as a “pioneer study” for tailor-made medicines [20]. This system may ultimately provide valuable contributions to medical treatment as well as basic research into the mechanisms of drug resistance in CML cells.

Ethical approval

The clinical trial included in this study was reviewed and approved by the Institutional Review Board of the Hokkaido University Graduate School of Medicine and by the relevant committees of all enrolled hospitals. All patients enrolled in those studies provided informed consent before collection of bone marrow or peripheral blood samples.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

During the preparation of this review article, our precious laboratory member and a contributor to this project, Ms. Akiko Kaneyasu, passed away. We would like to pray sincerely for the repose of her soul. The authors would like to also thank Ms. Ayumi Kikuchi for technical assistance and all members of Ohba laboratory for helpful discussions.

References

[1] Morise H, Shimomura O, Johnson F, Winant J. Intermolecular energy transfer in the bioluminescent system of Aequorea. Biochemistry 1974;13:2656–62.
[2] Prasher D, Eckenrode V, Ward W, Prendergast F, Cormier M. Primary structure of the Aequorea victoria green-fluorescent protein. Gene 1992;111:229–33.
[3] Heim R, Prasher DC, Tsien RY. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA 1994;91:12501–4.
[4] Yarbrough D, Wachter RM, Kallio K, Matz MV, Remington SJ. Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-A resolution. Proc. Natl. Acad. Sci. USA 2001;98:462–7.
[5] Mizutani T, Kondo T, Darmanin S, Tsuda M, Tanaka S, Tobiume M, et al. A novel FRET-based biosensor for the measurement of BCR-ABL activity and its response to drugs in living cells. Clin. Cancer Res 2010;16:3964–75.
[6] Ohba Y, Matsuda M. Visualization of signal transduction of Ras. Seikagaku 2004;76:16–28.
[7] Kerppola T. Visualization of molecular interactions by fluorescence complementation. Nat Rev Mol Cell Biol. 2006;7:449–56.
[8] Tsutsumi K, Fujioka Y, Tsuda M, Kawaguchi H, Ohba Y. Visualization of Ras-PI3K interaction in the endosome using BiFC. Cell Signal 2009;21:1672–9.
[9] Fujioka Y, Tsuda M, Hattori T, Sasaki J, Sasaki T, Miyazaki T, et al. The Ras-PI3K signaling pathway is involved in clathrin-independent endocytosis and the internalization of influenza viruses. PLoS ONE 2011;6:e16324.
[10] Matlin KS, Reggio H, Helenius A, Simons K. Infectious entry pathway of influenza virus in a canine kidney cell line. J Cell Biol. 1981;91:601–13.
[11] Doxsey SJ, Brodsky FM, Blank GS, Helenius A. Inhibition of endocytosis by anti-clathrin antibodies. Cell 1987;50:453–63.
[12] Chen C, Zhuang X. Epsin 1 is a cargo-specific adaptor for the clathrin-mediated endocytosis of the influenza virus. Proc. Acad. Sci. USA 2008;105:11790–5.
[13] Sieczkarski S, Whittaker G. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. J. Virol. 2002;76:10455–64.
[14] Fujioka Y, Tsuda M, Nanbo A, Hattori T, Sasaki J, Sasaki T, et al. A Ca(2+)-dependent signalling circuit regulates influenza A virus internalization and infection. Nat. Commun. 2013;4:2763.
[15] Fujioka Y, Nanbo A, Nishide S-Y, Ohba Y. Fluorescent protein-based biosensors to visualize signal transduction beneath the plasma membrane. Anal. Sci. 2015;31:267–74.
[16] Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. New Engl. J. Med. 2006;355:2408–17.
[17] Monitoring treatment and survival in chronic myeloid leukemia. Italian Cooperative Study Group on Chronic Myeloid Leukemia and Italian Group for Bone Marrow Transplantation. J. Clin. Oncol. 1999;17:1858–1868.
[18] Förster T. Zwischenmolekulare Energiewanderung und Fluoreszenz - Förster – 2006 - Annalen der Physik - Wiley Online Library. Ann. Der Phys. 1948.
[19] Miyawaki A. Visualization of the spatial and temporal dynamics of intracellular signaling. Dev. Cell 2003;4:295–305.
[20] Lu S, Wang Y. Fluorescence resonance energy transfer biosensors for cancer detection and evaluation of drug efficacy. Clin. Cancer Res. 2010;16:3822–4.

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