

Review Article

Fluorescence resonance energy transfer imaging of cell signaling from *in vitro* to *in vivo*: Basis of biosensor construction, live imaging, and image processing

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The progress in imaging technology with fluorescent proteins has uncovered a wide range of biological processes in developmental biology. In particular, genetically-encoded biosensors based on the principle of fluorescence resonance energy transfer (FRET) have been used to visualize spatial and temporal dynamics of intracellular signaling in living cells. However, development of sensitive FRET biosensors and their application to developmental biology remain challenging tasks, which has prevented their widespread use in developmental biology. In this review, we first overview general procedures and tips of imaging with FRET biosensors. We then describe recent advances in FRET imaging – namely, the use of optimized backbones for intramolecular FRET biosensors and transposon-mediated gene transfer to generate stable cell lines and transgenic mice expressing FRET biosensors. Finally, we discuss future perspectives of FRET imaging in developmental biology.

Key words: fluorescence resonance energy transfer, *in vivo* imaging, intravital imaging, two photon microscopy.

Introduction

Over the last few decades, the discovery of green fluorescent proteins (GFPs) has contributed to great progress of the life sciences, including cell biology and developmental biology (Giepmans *et al.* 2006). The fluorescent proteins (FPs) and their applications have become increasingly important and indispensable for exploring intracellular events in living organisms. The main advantage of FPs over chemical fluorescent dyes is that the former are genetically encoded, which allows them to be easily and stably expressed in cells and animals by conventional gene transfer techniques.

Förster (fluorescence) resonance energy transfer (FRET), is a radiationless energy transfer process from a donor fluorophore to an acceptor fluorophore placed in close proximity to the donor (Jares-Erijman & Jovin 2003). The energy transfer efficiency E is given by

$$E = \frac{(R_0/r)^6}{1 + (R_0/r)^6}; R_0^6 = c_0 \kappa^2 J n^{-4} Q_0 \quad (1)$$

where r is the distance between donor and acceptor; R_0 is the Förster distance where the FRET efficiency becomes 0.5; κ^2 is a factor determined by the relative orientation of transition moments of the donor and acceptor; J is the overlap of the donor emission and acceptor excitation spectra; n is the refractive index; Q_0 is the quantum yield of the donor; and $c_0 = 8.8 \times 10^{-28}$ for R_0 in nm (Jares-Erijman & Jovin 2003). In practical terms, the FRET efficiency depends on r , κ^2 , and J (Fig. 1a–c). With the advent of FPs, genetically-encoded biosensors based on the principle of FRET have been developed and used to monitor the spatio-temporal dynamics of intracellular signaling molecules such as ions (Miyawaki *et al.* 1997; Nagai *et al.* 2004), lipids (Sato *et al.* 2003), small GTPases (Mochizuki *et al.* 2001; Itoh *et al.* 2002), kinases (Kurokawa *et al.* 2001; Zhang *et al.* 2001), proteases (Takemoto *et al.* 2003), and so on (for more detail, see (Kiyokawa *et al.* 2011)).

Here, we overview the design and construction of genetically-encoded FRET biosensors, and describe

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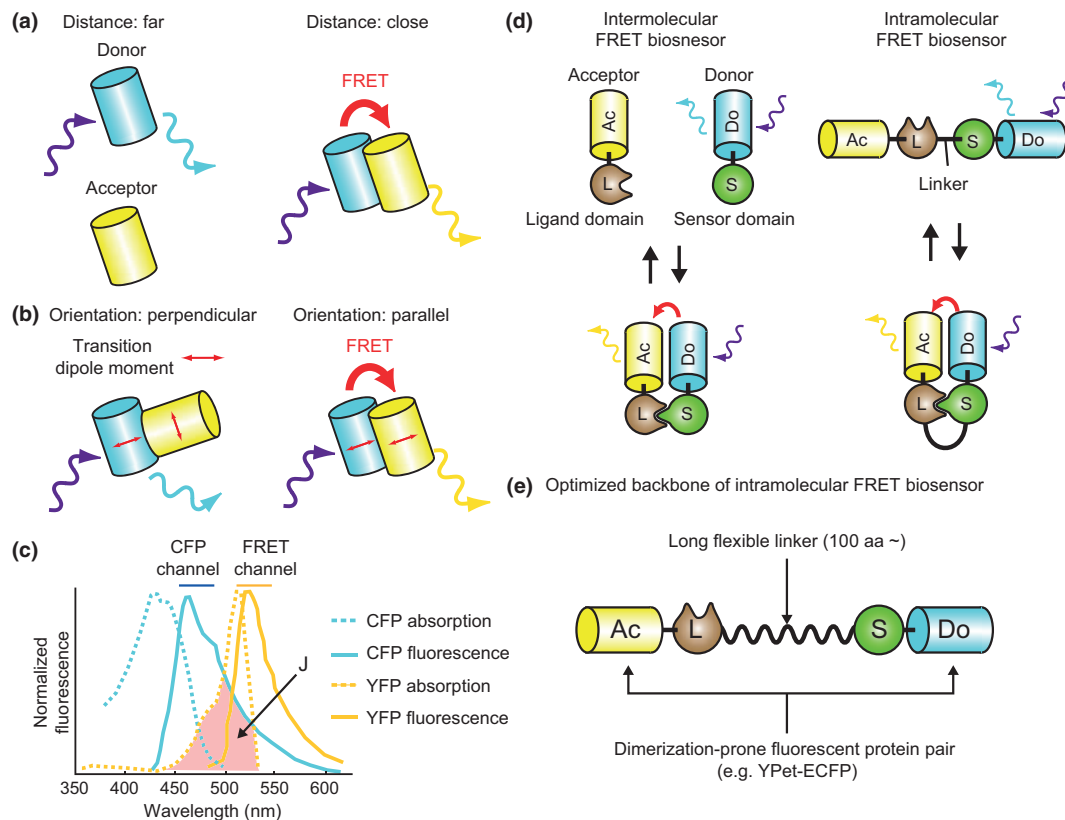


Fig. 1. The principle of fluorescence resonance energy transfer (FRET). FRET efficiency is dependent on the distance (a), the relative orientation (b) of the donor and acceptor fluorophore, and the overlap of the donor emission spectrum and acceptor excitation spectrum (c). (d) The two types of FRET biosensors: intermolecular (left) and intramolecular (right) FRET biosensors. The sensor domain senses the alteration in environmental signal, which induces conformational change of the sensor domain. The ligand domain recognizes the conformational change of the sensor domain, leading to the change in FRET efficiency. (e) An optimized backbone of an intramolecular FRET biosensor based on the Eevee system is shown.

recent advances in FRET biosensor construction and the generation of transgenic mice expressing FRET biosensors.

Structure and construction of FRET biosensors

FRET biosensors are classified into two categories, intermolecular-type and intramolecular-type biosensors (Fig. 1d). The intermolecular FRET biosensors consist of a protein fused with a donor fluorescent protein and a protein fused with an acceptor fluorescent protein. When the two proteins associate with each other, FRET takes place between these fluorescent proteins (Fig. 1d). This intermolecular FRET system can be easily set up, because the preparation of two plasmids expressing proteins fused with FPs is sufficient for FRET imaging. However, there are also considerable flaws in this system, such as the requirement of corrections and perturbation to the endogenous signaling (Miyawaki 2003, 2011). On the other hand, an intramolecular FRET biosensor usually suffers less from

these problems (Miyawaki 2003; Aoki & Matsuda 2009). As a trade-off, the developers usually need to spend a rather lengthy period for the design, construction, and evaluation of the intramolecular FRET biosensor. Nevertheless, through the effort of many laboratories, the number of intramolecular FRET biosensors has been steadily increasing. At the same time, due to the ease of the gene transfer and the high sensitivity of the new biosensors, the use of these tools has also been rapidly increasing.

Development of an optimized backbone for intramolecular FRET biosensors

In addition to the pair of FPs, a typical intramolecular FRET biosensor is comprised of sensor and ligand domains, which are connected by a linker. The aforementioned challenge in the development of intramolecular FRET biosensors resides in the assembly of these parts into a functional biosensor. The main reason for this difficulty is that both the distance and orientation

of donor and acceptor FPs affect the increase in FRET of intramolecular biosensors, but we cannot predict the 3D structure of the FRET biosensor in most cases. In particular, the contribution of the orientation-dependent FRET is almost impossible to foresee. Hence, intramolecular FRET biosensors have so far been developed and improved through multiple rounds of trial-and-error. To overcome this problem, we recently invented an optimized backbone of intramolecular FRET biosensors (Komatsu *et al.* 2011). The key concept in the optimized backbone is to minimize the orientation-dependent FRET and to render the biosensors completely “distance-dependent.” We call this system Eevee (extension for enhanced visualization by evading extra FRET). In this system, in an attempt to eliminate orientation-dependent basal FRET signal, we exploited a long flexible linker ranging from 116 to 244 amino acids in length, resulting in the increase in the gain of the FRET biosensors (Fig. 1e). Furthermore, a dimerization-prone FP pair for the donor and acceptor, for example, YPet and enhanced cyan fluorescent protein (ECFP), was used to increase the gain of the FRET signal in the Eevee system. Consequently, for the development of an intramolecular FRET biosensor, we only need to consider the combination of the ligand and sensor domains with the backbone optimized for the intramolecular FRET in the Eevee system.

As an example, we adduce the construction of a FRET biosensor for a Serine/Threonine kinase belonging to the CMGC-family, which includes cyclin-dependent kinase (CDKs), mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK), and CDK-like kinases. Because CMGC kinases preferably phosphorylate Ser or Thr residues followed by Pro (Ser/Thr-Pro), the WW domain, which recognizes phospho-Ser-Pro or phospho-Thr-Pro peptide, can be conveniently used as the ligand domain. For the other kinases, we use the FHA1 domain, which binds to the phospho-Thr-X-X-Asp motif. Therefore, a phosphorylated peptide as a sensor domain is designed to fit the recognition motif. The substrate sequences of the target kinase are used as the sensor domain, but they must still be carefully selected and evaluated in a trial-and-error fashion in order to determine whether the kinase will efficiently phosphorylate the peptide within the biosensor and thus obtain a highly sensitive and selective FRET biosensor.

Characterization of FRET biosensors in cultured cells

After the construction of the expression plasmid, the biosensor has to be validated to determine whether it faithfully monitors the activity of the target kinase. To

this end, we usually use cultured cells such as HeLa cells, which are easily transfected with plasmids by conventional lipofection methods.

We recommend that FRET biosensors be carefully evaluated and characterized in the following ways. (i) Using a positive control for FRET imaging: the FRET biosensors should be well characterized in order to check whether FRET imaging is proceeding appropriately in the microscopic setting. For example, we showed clear FRET images in HeLa cells expressing a protein kinase A (PKA) biosensor, AKAR3EV, upon stimulation with membrane permeable cAMP, dibutyl cAMP (dbcAMP) (Fig. 2a,b). Cells that express extremely high or low levels of biosensors should not be selected. Furthermore, care should be taken in regard to the photobleaching of YFP, which leads to an arti-

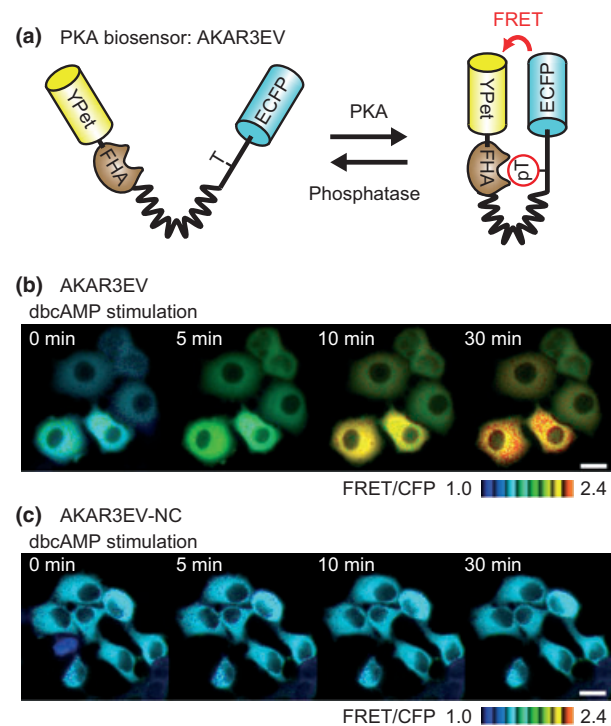


Fig. 2. Fluorescence resonance energy transfer imaging in cultured cells. (a) Structure of a protein kinase A (PKA) biosensor, AKAR3EV. (b) PKA activity in HeLa cells as visualized by AKAR3EV. HeLa cells expressing AKAR3EV were stimulated with 1 mmol/L dibutyl cAMP (dbcAMP). The fluorescence resonance energy transfer (FRET)/cyan fluorescent protein (CFP) ratio was gradually increased upon dbcAMP stimulation, indicating PKA activation. (c) A phosphorylation-deficient mutant of AKAR3EV, AKAR3EV-NC, was used as the negative control of AKAR3EV. HeLa cells expressing AKAR3EV-NC were stimulated with 1 mmol/L dbcAMP, which resulted in no increase in FRET/CFP. The colored side bars represent the upper and lower ranges of the FRET/CFP value. Scale bars, 20 μ m.

cial decrease of FRET during imaging. For more details of FRET imaging with cultured cells, please refer to the previous publications (Nakamura *et al.* 2005; Aoki & Matsuda 2009). (ii) Using a negative control: Several factors might affect the FRET signal irrespective of the intracellular signaling states. Hence, we have to assess whether the FRET change is associated with the event that we expect. The best way to clarify the mode of action of FRET biosensors is to use a negative control. Adducing Ser/Thr kinase FRET biosensor as an example, a mutant biosensor, in which the phospho-acceptor Ser or Thr is replaced with Ala, works best as a negative control (Fig. 2c). (iii) Validation by specific stimuli: The biosensors are eventually validated by specific ligands, inhibitors, or dominant active/negative mutants, if available. The verification by biochemical methods also provides a quantitative relationship between FRET increase/decrease and net change of intracellular signaling.

Establishment of stable cell lines expressing FRET biosensors by transposon-mediated gene transfer

The establishment of cell lines expressing intramolecular FRET biosensors has proven quite difficult using the conventional gene-transfer methods, such as lipofection of linearized plasmids or retrovirus-mediated gene transfer; therefore, the use of intramolecular FRET biosensors has been restricted mainly to cultured cells with a transient expression system. Recently, we circumvented this limitation by using a transposon-mediated gene transfer system (Komatsu *et al.* 2011; Aoki *et al.* 2012). In the transposon-donor plasmid, the cDNA gene encoding the FRET biosensor, drug-resistance gene, promoter, and poly-A signal should be sandwiched between transposon-specific inverted terminal repeat sequences (ITRs) (Fig. 3a). The plasmid is co-transfected with an expression plasmid of a transposase. Several days after transfection, the transfected cells are selected by antibiotics. To date, we have succeeded in establishing cultured cell lines stably expressing intramolecular FRET biosensors with *piggyBac* (Ding *et al.* 2005; Yusa *et al.* 2009) and *Tol2* (Kawakami & Noda 2004; Sumiyama *et al.* 2010) transposon systems. A good example of the use of these stable cell lines is the assessment of chemical drugs in living cells. HeLa cells expressing a FRET biosensor for extracellular signal-regulated kinase (ERK) MAP kinase were seeded onto glass-bottomed 96-well dishes, and 1 day after seeding, the cells were treated with serial dilutions of kinase inhibitors (Fig. 3b). It should be noted that we were able to evaluate the effect of these inhibitors on ERK activity in living cells at the single cell level using this system.

Intravital imaging with transgenic mice expressing FRET biosensors by two-photon excitation microscopy

The transposon-mediated gene transfer technique was also applicable for the generation of transgenic mice expressing FRET biosensors (Kamioka *et al.* 2012). Cytoplasmic injection of the *Tol2*-transposon-donor plasmid and *Tol2* transposase mRNA causes highly efficient integration of the transgene in comparison to the conventional methods (Sumiyama *et al.* 2010; Kamioka *et al.* 2012). We have already generated transgenic mice with heritable and functional biosensors for PKA and ERK (Kamioka *et al.* 2012).

The availability of transgenic mice expressing FRET biosensors prompted us to visualize protein kinase activity in living animals by intravital imaging. For this purpose, we used two-photon excitation microscopy (TPEM), which allows deeper tissue penetration of the light and less phototoxicity than the conventional single-photon confocal microscopy (Molitoris & Sandoval 2005). Intravital imaging with TPEM has been applied to a wide range of tissues and organs in mice, including the brain (Noguchi *et al.* 2011; Grienberger & Konnerth 2012), auricular skin, blood vessels (Egawa *et al.* 2011; Kamioka *et al.* 2012), lymph nodes (Okada *et al.* 2005; Cahalan & Parker 2008; Kitano *et al.* 2011), cecum (Toiyama *et al.* 2010; Tanaka *et al.* 2012), intestines (Mcdole *et al.* 2012), bone (Ishii *et al.* 2009), lung (Looney *et al.* 2011), liver, pancreas, kidney (Ashworth *et al.* 2007; Camirand *et al.* 2011), heart (Li *et al.* 2012), muscles (Cao *et al.* 2012), and cancers (Kedrin *et al.* 2007; Le Devedec *et al.* 2011). Here, we will briefly review general procedures and tips for intravital FRET imaging with TPEM, although the techniques required for TPEM are heavily dependent on the particular tissues or organs targeted. First, the mice are anesthetized with volatile anesthetic isoflurane. Next, the target tissue is exposed by hair removal and/or surgical operation. The mice are placed on a microscope stage maintained at 30°C using a heating pad. To minimize motion artifacts ascribed to heart-beating and breathing of mice, the object to be imaged should be moved away from sources of motion, such as the heart (Fig. 4b). Laser ablation or intravenous administration of drugs is easily applied for *in vivo* perturbation under the condition of intravital imaging (Fig. 4c,d). For FRET imaging with TPEM, we use a wavelength of 840 nm in two-photon excitation for CFP. Importantly, although we do not know the reason at this moment, a slow scan speed (10–100 us/pixel) and a low voltage of the photomultiplier tube decrease the noise of CFP and FRET signals, resulting in an increase in the signal-to-noise ratio of the FRET/CFP value, which is a quantitative measure of FRET

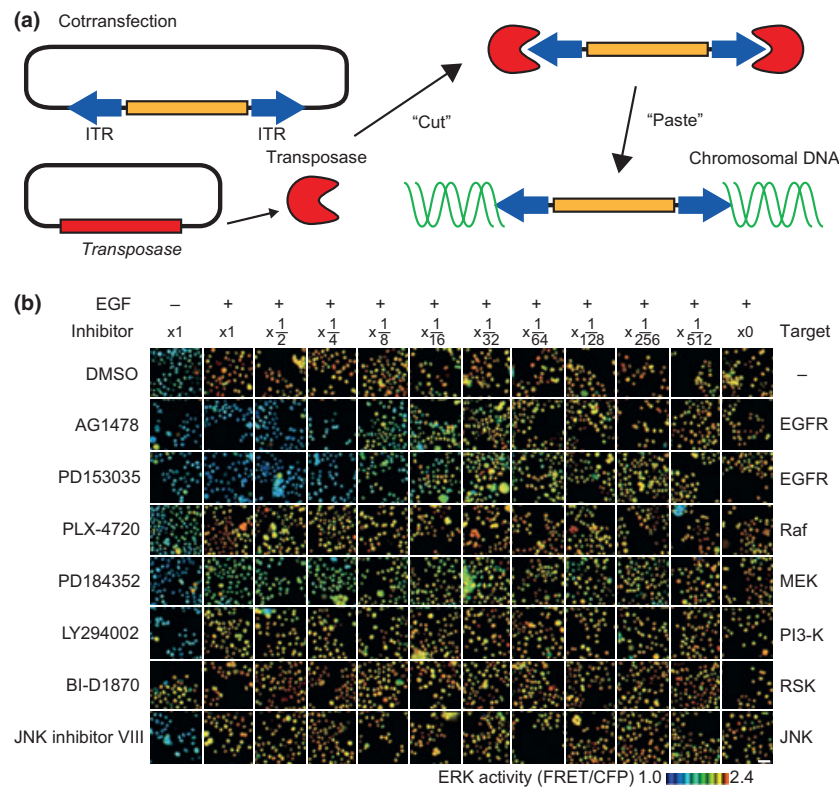


Fig. 3. Fluorescence resonance energy transfer-based assay of extracellular signal-regulated kinase (ERK) activity to several kinase inhibitors with stable cell line. (a) Scheme of the transposon-mediated gene transfer. The cells are co-transfected with two plasmids; one is an expression plasmid of the transposase, and the other is a plasmid encoding a gene of interest that is sandwiched with transposase-specific internal terminal repeat (ITR). The transposase cuts and pastes the gene of interest into chromosomal DNA. (b) HeLa cells stably expressing a fluorescence resonance energy transfer (FRET) biosensor for ERK as nucleus, EKAREV-nuc, were plated on a 96-well plate, and then treated with (+) or without (-) 25 ng/mL epidermal growth factor (EGF) and serial dilutions of kinase inhibitors. The cells were imaged with an epifluorescence microscope. The kinase inhibitors and their target kinases were as follows: 1 μ mol/L AG1478 (EGF receptor [EGFR] inhibitor); 1 μ mol/L PD153035 (EGFR inhibitor); 10 μ mol/L PLX-4720 (Raf inhibitor); 10 μ mol/L PD184352 (MEK inhibitor); 10 μ mol/L LY294002 (PI3-K inhibitor); 10 μ mol/L BI-D1870 (RSK inhibitor); and 10 μ mol/L JNK inhibitor VIII (JNK inhibitor). The colored bar represents the upper and lower ranges of the FRET/cyan fluorescent protein (CFP) value. Scale bar, 50 μ m.

images (see below). It should be noted, however, that slow scan speed makes the image sensitive to motion effect of heart beat or breathing, and also causes generation of heat and photo-bleaching. Therefore, slowing the scan speed is a trade-off of these disadvantages. In general, long intervals of time-lapse imaging avoids heat generation. Intravital imaging appears to be more resistant to heat generation than *ex vivo* and *in vitro* imaging, because of the existence of blood circulation. The problem of photo-bleaching should be examined carefully by trial-and-error to see whether photo-bleaching takes place in that microscopic setting. A simple way is the reduction of laser power. This could be achievable, because the slow scan speed makes it possible to detect more photons than fast scan speed.

For successful intravital imaging with TPEM, it is important to maximize the collection efficiency of

scattering fluorescence generated at the focal point of two-photon excitation. In order to take full advantage of the depth penetration of TPEM, the use of external detectors, which are pinhole-less detectors located close to the specimen to reduce the optical elements and light path length, and an objective lens with the highest numerical aperture and field number, are best suited. This leads to the question of whether an upright microscope or an inverted microscope is preferable for intravital imaging with TPEM. In fact, each microscope has its advantages and disadvantages. With the upright microscope, micropipettes are accessible to the observed area of the specimen. Currently, objective lenses customized for TPEM, that is, lenses with high numerical apertures, high field numbers and optical transparency for infra-red light, are usually designed for the upright microscope. However, it is difficult to stabilize the animal under the upright

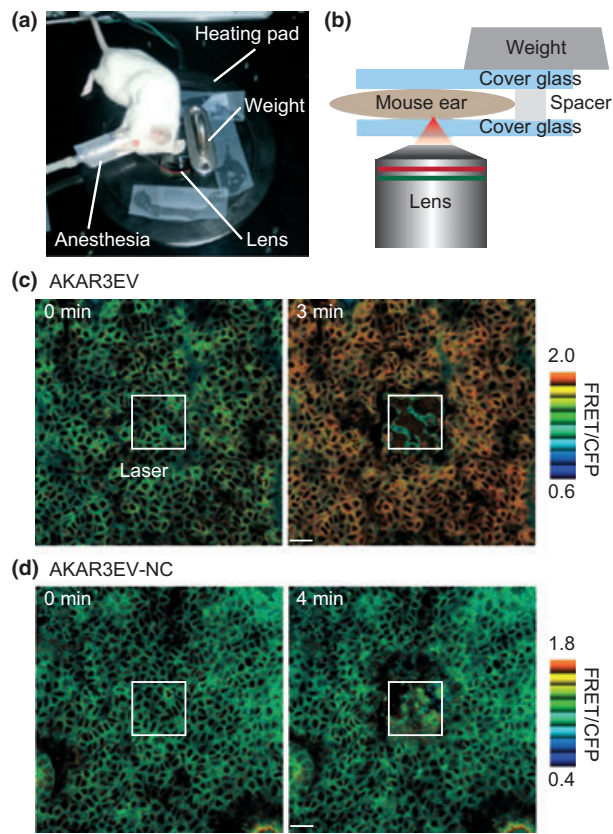


Fig. 4. Intravital imaging with transgenic mice expressing a fluorescence resonance energy transfer (FRET) biosensor. (a) An anesthetized mouse positioned on the heating pad of the stage in an inverted microscope. (b) Diagram of an auricular skin sample prepared for two-photon excitation microscopy (TPEM). (c) and (d) The auricular skin sample of transgenic mice expressing AKAR3EV (c) or AKAR3EV-NC (d) was observed by TPEM. Laser ablation was performed in the region demarcated by the white box. The colored side bars represent the upper and lower ranges of the FRET/cyan fluorescent protein (CFP) value. Scale bars, 20 μ m.

microscope, and therefore samples are susceptible to motion artifacts. On the other hand, with the inverted microscope, the animals can be held down on the cover glass, minimizing motion artifacts. These advantages and disadvantages for upright and inverted microscopes should be considered carefully.

Image processing and quantification of FRET imaging

We usually use MetaMorph software for the image analysis. However, other free software packages, such as ImageJ (<http://rsb.info.nih.gov/ij/>), are also applicable to analyze these images. The background signals are subtracted in each plane of FRET and CFP images. The time-course of the change in the FRET/CFP ratio is calculated as follows: A region of interest

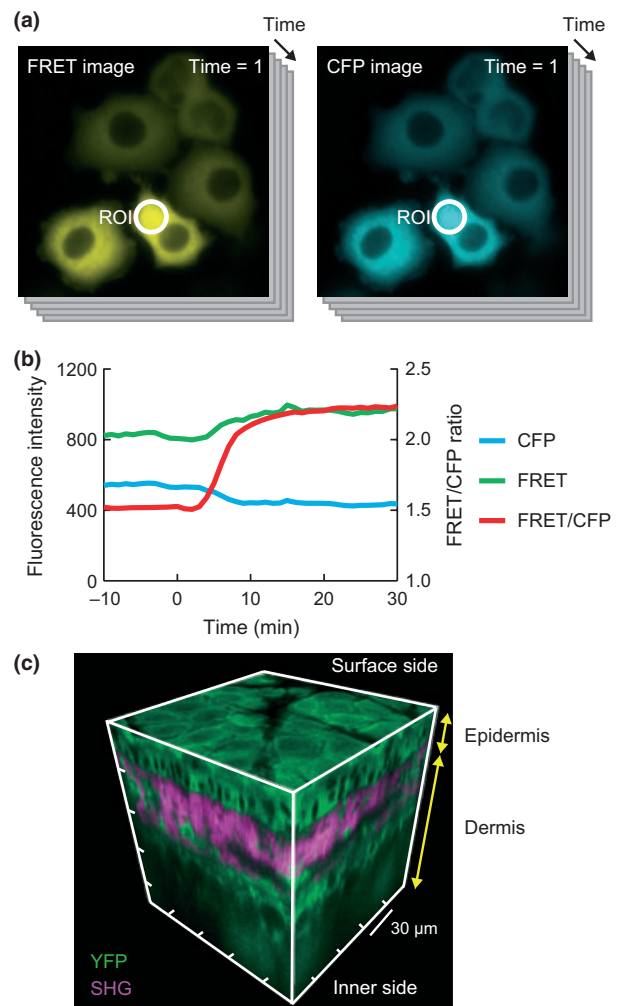


Fig. 5. Image data processing. (a) A region of interest is positioned on a target cell in stack files of fluorescence resonance energy transfer (FRET) image and cyan fluorescent protein (CFP) images. The fluorescence intensities of FRET and CFP are measured and exported. (b) The time course of FRET signal (green line), CFP signal (blue line), and FRET/CFP ratio (red line) are plotted as a function of time after dibutyl cAMP (dbcAMP) stimulation. (c) A reconstituted 3D structure of an auricular skin sample from a transgenic mouse expressing AKAR3EV by Imaris software. The localizations of AKAR3EV (green) and collagen (magenta) were observed by two-photon excitation fluorescence and second harmonic generation (SHG) microscopy, respectively.

(ROI) is set on a cell of interest in FRET and CFP images (Fig. 5a). Then, the signal intensities of FRET and CFP are exported to a file formatted by ASCII or Microsoft Excel. The data are then processed to draw line graphs in Excel software (Fig. 5b). The increase in FRET accompanies the inverse correlation between the fluorescence intensities of FRET and CFP signals. With a function in MetaMorph software, FRET/CFP ratio images are reconstructed as an intensity modu-

lated display (IMD) mode (Tsien & Harootunian 1990). The IMD mode uses the FRET/CFP ratio to determine the color hue, and the intensity of CFP, YFP, or average thereof to determine the intensity of the color hue of each pixel. In the IMD mode, we can see not only the FRET signal but also the distribution of the biosensor in the cells. Many literatures using IMD (eight bits) adopt eight ratio colors (three bits) and 32 intensities (five bits) for each pixel, which is reasonable empirically. Theoretically, the signal-to-noise (S/N) ratio of FRET/CFP is smaller than the S/N ratio of original FRET and CFP images, because of noise propagation. For example, assuming that S/N ratio of FRET is equal to that of CFP image, the S/N ratio of FRET/CFP ratio image is reduced to approximately 70% of that of the FRET and CFP image. Therefore, the bit number of FRET/CFP used to determine the color hue could be smaller than that of the FRET or CFP image used to determine the intensity of color hue. The 16 ratio colors (four bits) and 16 intensities (four bits) mode is also useful, when subtle changes of ratio are emphasized rather than visualizing morphology.

Future perspectives of FRET imaging

FRET biosensors visualize the activity of signaling molecules in high spatial and temporal resolutions. These quantitative data are driving the progress of systems biology (Kamioka *et al.* 2010; Tay *et al.* 2010; Aoki *et al.* 2011). Moreover, the ability to establish stable cell lines and transgenic mice expressing FRET biosensors provides a good tool to evaluate the effects of drugs on the pharmacodynamics and toxicity in living cells and animals, respectively. At the present time, one of the limiting factors for these imaging modalities is the development of a 3D image processing program or algorithm. Imaris and Volocity softwares are commonly used for 3D image visualization and analysis such as cell tracking. Figure 5c represents a 3D image of skin imaged by TPEM. Note that the second harmonic generation (SHG) visualizes the dermal collagenous matrix (magenta in Fig. 5c). However, unlike in the case of cultured cells, the image processing algorithm for extracting 3D quantitative characteristics still awaits important improvements. Although there are numerous problems to be overcome in the future, the advance of fluorescence imaging continues to expand our understanding of developmental biology.

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