Fluorescent Biosensors

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A Tunable FRET Circuit for Engineering Fluorescent Biosensors**

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Biosensors based on fluorescence resonance energy transfer (FRET) have become powerful tools for monitoring molecular events in living systems.[1] As new assay platforms are developed,[2] these biosensors should find new applications in high-throughput drug screening, activity profiling, and mechanistic studies. For engineering such biosensors, a FRET pair[3] for which the emission of the donor fluorophore overlaps the excitation of the acceptor fluorophore is required. The degree of spectral overlap is an important determinant for the efficiency of FRET. On the other hand, both the excitation and emission spectra of the donor and acceptor should be separated to avoid cross excitation and simplify detection. Such requirements limit the choices of FRET pairs even when the family of fluorescent proteins is rapidly expanding.[4] The ability to choose fluorophores based on their individual spectral properties beyond the requirements for FRET as well as the versatility of tuning FRET efficiency should open new avenues in engineering FRET-based biosensors. Such a method could lead to biosensors with high signals or with varying spectral properties for use in parallel with other fluorescent molecules. We theorized that two fluorophores without optimal spectral overlap could be utilized when they are coupled together by an intermediate fluorophore. Excitation of the donor would lead to energy transfer to the intermediate fluorophore, which transfers energy to the acceptor fluorophore, as shown in other systems.[5] Owing to the presence of the intermediate fluorophore, or "tuner", and this sequential energy transfer, the efficiency of the FRET circuit can be tuned by changing the properties of the tuner.

To evaluate the use of a FRET circuit in the construction of fluorescent biosensors, we chose to build a new A-kinase activity reporter (AKAR) with cyan–red ratiometric readout. AKAR[6] measures activity of cAMP-dependent protein kinase (PKA), and usually consists of a FRET pair, a phosphoamino acid binding domain, and a PKA substrate. When phosphorylated by PKA, intramolecular binding of the substrate by the phosphoamino acid binding domain drives conformational reorganization, leading to an increase in FRET. Cyan fluorescent protein (CFP) and red fluorescent protein (RFP), particularly mCherry,[7] have separate emissions but limited spectral overlap, and are considered a poor FRET pair.[8] In the new CRY AKAR (CRY = cyan–red–yellow), a yellow fluorescent protein (YFP), mVenus, was chosen as the tuner based on its appropriate spectral overlap with CFP and RFP. The phosphorylation-dependent conformational switch composed of Forkhead associated domain 1 (FHA1) and PKA substrate was sandwiched between Cerulean,[9] a CFP, and mCherry, linked to mVenus through a short spacer (Figure 1a).

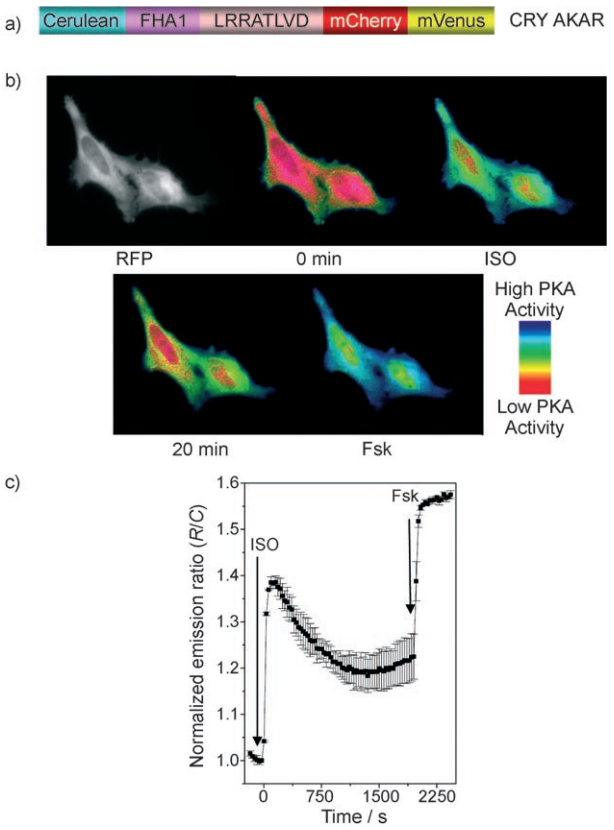

Figure 1. Development of an AKAR with a tunable FRET circuit. a) Domain structures of CRY AKAR. b) RFP fluorescence image and pseudocolor time course of two HeLa cells expressing CRY AKAR treated with ISO and Fsk. c) A representative time course of HeLa cells expressing CRY AKAR treated with ISO followed by treatment with Fsk. *R* = red emission intensity, *C* = cyan emission intensity.

To test the presence of a FRET circuit, we calculated efficiencies for the energy-transfer processes in CRY AKAR. Energy transfer from Cerulean to mCherry has two potential paths, a direct path from Cerulean to mCherry and a

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sequential path from Cerulean to mVenus, then mVenus to mCherry. To determine the efficiency of each transfer, HEK-293 cells transiently transfected with either CRY AKAR or a similar construct without mVenus were subject to a series of photobleaching procedures. First, cells expressing CRY AKAR underwent RFP photobleaching. At this point, recovery of YFP fluorescence was measured to obtain the FRET efficiency between mVenus and mCherry: 36(±2)% ($n=5$, where n is the number of cells). With mCherry fluorophores irreversibly photobleached, FRET occurs only from Cerulean to mVenus. The same cells then underwent YFP photobleaching, and CFP recovery was measured to calculate the FRET efficiency between Cerulean and mVenus in the absence of mCherry: 19(±3)% ($n=5$). Next, we determined the efficiency of direct energy transfer from Cerulean to mCherry in the absence of mVenus by using the two-fluorophore construct. RFP was photobleached, and CFP recovery was measured to yield a FRET efficiency of 18(±7)% ($n=4$).

With these three FRET efficiencies, it is straightforward to calculate[8a] the percentage of sequential versus direct energy transfer from Cerulean to mCherry (see the Supporting Information). The percentages of sequential and direct energy transfer were found to be approximately 29% and 71%, respectively. Thus, this FRET circuit is functional, with mVenus acting as the tuner in the sequential energy transfer between Cerulean and mCherry, along with a direct energy-transfer path. As the efficiency of sequential energy transfer is largely determined by the FRET efficiency between mVenus and mCherry (see the Supporting Information), the current 36% leaves room for efficiency tuning, for example, by optimizing the spacer between mCherry and mVenus.

Next we tested the cellular responses of CRY AKAR. HeLa cells were transiently transfected and imaged using appropriate filter sets for cyan and red emissions during cyan excitation. As shown in Figure 1b, CRY AKAR diffused throughout the cytoplasm and somewhat into the nucleus. Stimulation with the beta-adrenergic receptor agonist isoproterenol (ISO) caused a decrease in cyan emission and increase in red emission (not shown). Using the ratio of red to cyan emissions as a readout, ISO stimulation yielded an average response of 39(±2)% ($n=5$; see the Supporting Information). After this response reversed following receptor desensitization, cAMP clearance, and sensor dephosphorylation,[6b,c] the same cells were treated with the transmembrane adenylyl cyclase activator forskolin (Fsk), yielding a more robust response from CRY AKAR (Figure 1c). The average response with Fsk stimulation was 54(±6)% ($n=5$), thus exceeding the dynamic range of AKAR3, currently the best CFP–YFP-based AKAR, which has a maximum response of about 40%.[6c]

CRY AKAR successfully tracks intracellular PKA activity with a cyan–red ratiometric readout. Its use in cellular studies should be compatible with the presence of yellow fluorescent compounds, which largely affect probes with a cyan–yellow readout. As validation, HeLa cells expressing CRY AKAR were treated with 50 μM L-sepiapterin, a GTP cyclohydrolase I inhibitor with CFP-like excitation and broad, predominantly yellow emission (Figure 2a). As shown in Figure 2b,

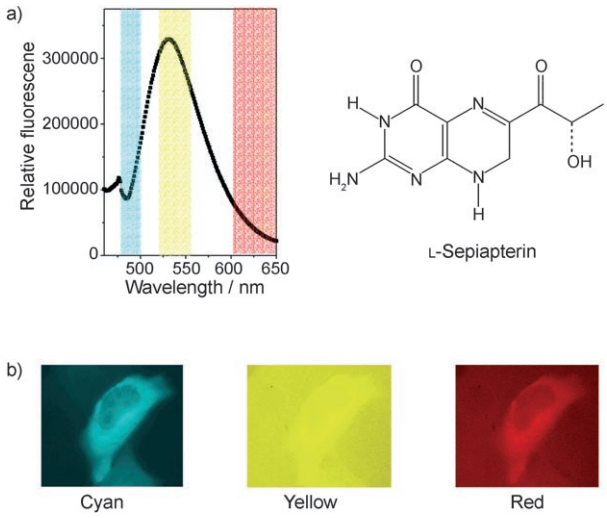

Figure 2. Cyan–red ratiometric readout of CRY AKAR allows simultaneous use of a yellow fluorescent compound L-sepiapterin, which cannot be used with the common CFP–YFP FRET pair. a) Emission spectrum of L-sepiapterin with excitation at 434 nm. b) Individual fluorescence images of CRY AKAR reflecting cyan, yellow, and red emission with CFP excitation in the presence of the fluorescent compound L-sepiapterin.

the presence of L-sepiapterin affected the detection of CFP minimally. However, the image of yellow fluorescence shows very little contrast between the transfected cell and background owing to compound fluorescence. Conversely, the transfected cell was clearly discernable with the detection of red emission upon cyan excitation. The Fsk-stimulated response was measured to be a 40% increase in the presence of L-sepiapterin, whereas the response cannot be measured using the cyan–yellow ratiometric readout of AKAR3. Thus, the ability to choose fluorophores with varying spectral properties in constructing FRET-circuit-based biosensors allows simultaneous use of fluorescent compounds that cannot be used otherwise. This finding is particularly useful as these fluorescent biosensors are utilized in cellular mechanistic studies or in high-throughput compound screening, where fluorescent compound interference is an important issue.[10]

In conclusion, we have demonstrated that in a FRET circuit containing Cerulean, mVenus, and mCherry, excitation of Cerulean leads to energy transfer to mCherry by both a direct and sequential path. "Tuning" the ratio of sequential versus direct energy transfer may be used to change the properties of the biosensor, for example, to maximize the change in FRET. Strategies for tuning such reporters include linker engineering and choosing tuners with different spectral properties. This tunable FRET system has led to the development of an improved AKAR with cyan–red ratiometric readout for more versatile use in live-cell analysis. The same FRET circuit should be applicable to other FRET-based biosensors, such as second-messenger indicators.[11] Furthermore, the concept of tunable FRET can be extended to other fluorophores and allows the use of two spectrally distant fluorophores as FRET donor and acceptor. The only requirement is that there exists a fluorophore that serves as a FRET

acceptor for the donor of choice and FRET donor for the acceptor of choice, which is spectrally distinguishable from both. When these criteria are met, fluorophores can be chosen for their unique spectral properties, which could assist in avoiding overlap with cell autofluorescence or drug fluorescence, or for high photostability in specific applications. The concept of tunable FRET should also be applicable to fluorescent dyes, quantum dots, and even bioluminescent proteins for tunable bioluminescence resonance energy transfer (BRET). Future experiments will test these possibilities to further expand the molecular tool kit for engineering biosensors for use in live-cell tracking of activities of biomolecules and in high-throughput drug screening.

Experimental Section

The genes encoding fluorescent proteins were PCR amplified using primers that introduce flanking restriction sites. PCR products then underwent restriction digestion followed by ligation into the appropriate sites in AKAR3.[6c] Complete genes for the new constructs were then subcloned into pCDNA3′ behind a Kozak sequence for mammalian expression.

HEK-293 or HeLa cells were plated onto sterilized glass coverslips in 35-mm dishes and grown to about 50% confluency in DMEM (10% fetal bovine serum (*m*/*v*) at 37°C with 5% CO_2). Cells underwent Fugene 6 mediated transfection and were allowed to grow for 12–24 h before imaging as described previously.[6] mCherry photobleaching was carried out with a 568DF55 excitation filter with 600DRLP dichroic mirror; cells were illuminated in this way for 25 min. mVenus photobleaching was carried out with a 525DF40 excitation filter with 600DRLP dichroic mirror; cells were illuminated for 5 min.

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