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论文题目： Development of a Genetically Encoded

Fluorescence Lifetime-based PKA Biosensor

Development of a Genetically Encoded Fluorescence Lifetime-based PKA Biosensor

Ruby Zhang

Abstract

Protein kinase A (PKA) is a central effector of cAMP signaling whose activity is dynamically regulated across subcellular compartments, thus requiring biosensors capable of quantitative monitoring with high spatiotemporal resolution. However, existing fluorescence-lifetime-based PKA biosensors are limited by relatively small lifetime responses, while fluorescence lifetime imaging microscopy (FLIM) itself presents challenges for high-throughput biosensor screening.

This study had two purposes: first, develop a genetically encoded, single-fluorophore PKA biosensor with an improved fluorescence-lifetime response; second, establish an efficient strategy for fluorescence-lifetime based biosensor high-throughput screening.

The biosensor designed in this study incorporated a PKA substrate peptide (PKA_{sub}), an FHA1 phosphopeptide-binding domain, and a circularly permuted Venus (cpVenus) as the fluorescence-lifetime reporter. To overcome the low throughput of conventional FLIM-based screening, a FRET-based screening platform was established by adding a dye-labeled HaloTag, dye425-HTL, to the cpVenus as an energy donor. Thus, candidates with desirable lifetime-related changes could be selected based on fluorescence-intensity measurements. A library of 108 linker-truncation combinations was designed, from which 97 reliable candidates were experimentally created and screened. The NΔ8/CΔ0 variant produced the largest lifetime response and was subsequently converted into the single-fluorophore FLIM-PKA biosensor by removing dye425-HTL.

Upon pharmacological activation of PKA, FLIM-PKA exhibited a fluorescence-lifetime change of -0.75 ± 0.04 ns, exceeding the responses of previous FLIM-based PKA biosensors; FLIM-PKA also showed partial signal recovery following PKA inhibition.

These results demonstrate that the study successfully fulfilled its purposes: developing a high-dynamic-range fluorescence-lifetime-based biosensor for PKA activity and

establishing a FRET-based high-throughput screening strategy for developing and optimizing genetically encoded FLIM biosensors.

Keywords: Protein kinase A (PKA); fluorescence lifetime imaging microscopy (FLIM); genetically encoded biosensor; high-throughput screening; Förster resonance energy transfer (FRET).

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1 Introduction

1.1 Background information

1.1.1 Protein kinases and Kinasopathies

Protein kinases, a large class of enzymes, transfer phosphate groups from high-energy donor molecules to amino acid residues on substrate proteins. Protein kinase genes account for approximately 2% of the human genome, comprising more than 500 kinase members¹. They regulate downstream target proteins by inducing phosphorylation-dependent conformational changes or by modifying protein-protein interactions that control the assembly and subcellular localization of protein complexes².

Kinasopathies are inherited or acquired diseases caused by abnormal protein kinase activity, including excessive activation and loss of function. Over the past two decades, kinase gene mutations have been closely associated with numerous human diseases, including developmental disorders, metabolic diseases, and specific cancers. Being restricted by selective pressure against the accumulation of variation, subtle changes in kinase structure can produce prominent phenotypic consequences³.

In cancer, aberrant kinase activation is a major pathogenic mechanism. For example, constitutively activating mutations in EGFR, such as L858R, contribute to non-small-cell lung cancer through continuously activating the RAS-MAPK pathway^{4,5}. On the other hand, loss of kinase function can bring severe consequences as well. Reduced LRRK2 kinase activity can impair lysosomal function, leading to abnormal α -synuclein aggregation, contributing to Parkinson's disease⁶. Current therapeutic strategies for kinasopathies have traditionally focused on small-molecule inhibitors, while newer strategies, including allosteric regulation, are being developed to improving therapeutic specificity⁷.

1.1.2 Protein kinase A (PKA)

In 1968, Donald Walsh and colleagues first isolated the cAMP-dependent protein kinase from rabbit skeletal muscle, now known as PKA⁸. This discovery not only clarified the molecular mechanism by which epinephrine regulates glycogenolysis through the cAMP-PKA pathway, but also established protein phosphorylation as the core of cellular signal transduction. PKA, a serine/threonine kinase, is a heterotetrameric holoenzyme composed of a regulatory-subunit dimer (R subunits) and two autoinhibited catalytic subunits (C subunits) in its inactive state. Binding of cAMP to the R subunits induces conformational changes, releasing the C subunit from

the holoenzyme and activating them, which subsequently phosphorylate downstream targets.

As a central effector of the cAMP signaling pathway, PKA has broad substrate specificity. PKA participates in diverse signaling pathways, ranging from glucose homeostasis to gene regulation. In neurons, PKA contributes to the regulation of neural function by modulating neuronal development, synaptic transmission, and synaptic plasticity⁹¹⁰. At the organismal level, PKA regulates learning, memory, and social behavior and is closely associated with neurodevelopmental disorders and psychiatric diseases¹¹¹².

PKA activation is tightly connected to G protein-coupled receptor (GPCR) signaling. As illustrated in Fig. 1-1, ligand binding to a transmembrane GPCR induces a conformational change in the G protein and promotes GDP-GTP exchange on the $G\alpha$ subunit, leading to dissociation or rearrangement of $G\alpha$ and the $G\beta\gamma$ dimer¹³. $G\alpha$ and $G\beta\gamma$ can subsequently interact with downstream targets separately to initiate specific signaling pathways. Dissociation of the $G\alpha_s$ subunit activates adenylyl cyclase and increases intracellular cAMP production. The elevated cAMP then binds the R subunit of PKA, releasing the C subunit and enabling the phosphorylation of downstream targets. The ligand-GPCR-cAMP-PKA cascade is thus formed. After GPCR-mediated activation of adenylyl cyclase, the intrinsic GTPase of the $G\alpha$ subunit hydrolyzes GTP to GDP, allowing $G\alpha$ to dissociate from adenylyl cyclase and terminate the signal initiated by ligand binding^{13,14}.

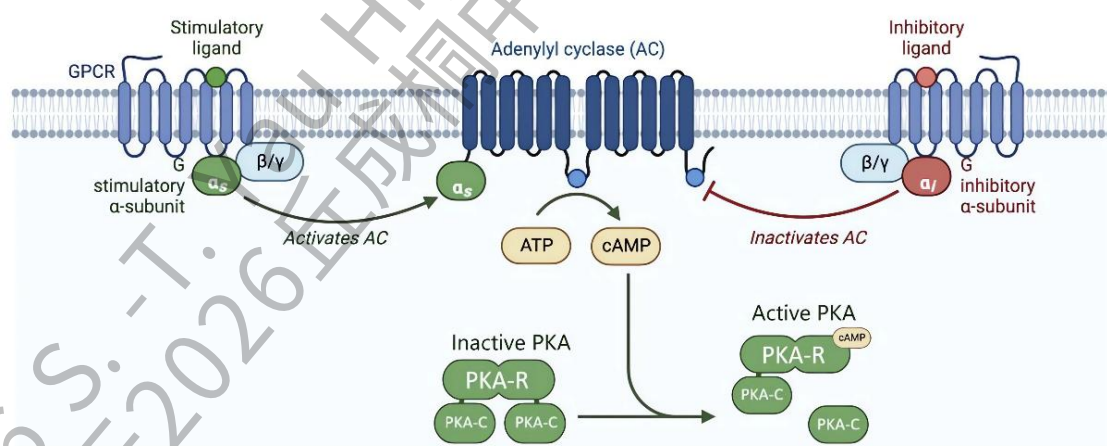


Figure 1-1. Illustration of GPCR-mediated regulation of PKA activity.

Because PKA has broad substrate specificity, cells achieve precise control of its function through two key mechanisms. First, spatial confinement of PKA is partly achieved through the binding of the R subunit and A-kinase anchoring proteins (AKAPs), which anchors PKA to specific subcellular regions. Second, local

phosphodiesterases (PDEs) regulate cAMP concentrations within specific compartments dynamically by degrading cAMP, precisely controlling the activity and spatiotemporal distribution of PKA. This mechanism ensures the targeted-effects of PKA across diverse signaling pathways, including metabolism and transcription, while preventing signal interference caused by nonspecific phosphorylation¹⁵⁻¹⁷. The precise regulation of intracellular PKA activity produces substantial spatiotemporal heterogeneity in PKA distribution and activity. Therefore, in order to study PKA regulation, tools capable of real-time quantitative measurement with high spatiotemporal resolution in living systems are needed.

1.1.3 Current methods for detecting PKA activity

1.1.3.1 Radioisotope labeling

Radioisotope labeling is a classical method for measuring kinase activity due to its high sensitivity and direct readout of substrate phosphorylation through the incorporation of radiolabeled phosphate. The assay is widely used for kinase activity measurements, kinetic characterization, and kinase inhibitor profiling. However, using radioactive materials requires rigorous protection and specialized waste disposal, raising safety concerns¹⁹.

1.1.3.2 ELISA

ELISA-based kinase assays measure kinase activity by quantifying the phosphorylated substrate generated using antibody-based detection. This method is commonly used for *in vitro* kinase assays. Compared with radioisotope labeling, ELISA is relatively safer and inexpensive. It can therefore be used for the screening of small-molecule kinase activators or inhibitors¹⁹. However, the performance of ELISA-based assays depends heavily on the specificity and sensitivity of the antibodies and requires careful optimization of conditions. It is also affected by buffer composition, concentration, and reaction time. Most importantly, it is unable to measure kinase activity in living systems real-time²⁰.

1.1.3.3 Genetically encoded fluorescent biosensors

In recent years, genetically encoded biosensors have enabled real-time monitoring in living cells, complex tissues, and brain regions, becoming powerful tools for analyzing signaling pathways²¹. These biosensors utilize properties of fluorescent proteins to reflect molecular events, allowing fluorescence changes to serve as readouts of cellular signaling.

The development of PKA activity monitoring began with two major advances. In

2000, the Nagai group developed ART, the first biosensor capable of real-time monitoring of the cAMP-PKA pathway in living cells, overcoming the limitations of conventional static measurements²². The following year, Jin Zhang and colleagues developed AKAR, which revealed a relationship between phosphorylation rate and substrate-kinase proximity through spatial targeting, establishing a framework for kinase-activity sensing²³.

Although AKAR1 pioneered compartment-specific PKA activity measurements, its low sensitivity to phosphatases resulted in irreversible FRET signals and limited physiological applications. In 2005, the Jin Zhang group replaced the phosphopeptide-binding domain 14-3-3 τ with the FHA1 domain to develop AKAR2, substantially improving sensor reversibility and sensitivity²⁴. In the following year, Allen and Zhang developed AKAR3 by incorporating a circularly permuted Venus (cpV-E172) as the FRET acceptor, increasing the sensor's dynamic range to a 31-41% change in the FRET emission ratio, approximately twice that of its predecessors²⁵.

Traditional FRET-based PKA biosensors are limited by spectral interference between two fluorescent proteins and constraints imposed by β -barrel structures on FRET efficiency, making multicolor imaging difficult and restricting dynamic range.

To overcome limitations of FRET-based sensors, Sohum Mehta and colleagues developed ExRai-AKAR in 2018, a single-fluorophore excitation-ratiometric biosensor that achieved a 143.7% increase in the 480/380-nm excitation ratio ($\Delta R/R$) upon PKA stimulation, thereby avoiding spectral-overlap problems²⁶. In 2021, Jin-Fan Zhang and colleagues optimized the linker region to develop ExRai-AKAR2, increased its dynamic range to an 869% 480/405-nm excitation-ratio response ($\Delta R/R$), approximately 20-fold higher than that of conventional FRET-based PKA sensors such as AKAR4, making it a powerful tool for detecting subtle changes in PKA activity²⁷.

1.2 Biosensors based on fluorescence lifetime imaging

Most genetically encoded kinase biosensors are based on fluorescence-intensity imaging, which is easily quantitative and possess effective high-throughput screening methods. However, results generated from this method can be affected by expression level, fluctuations in excitation intensity, and tissue scattering, making them less suitable for comparisons across samples or long-term quantitative measurements.

Fluorescence-lifetime-based biosensors can overcome these limitations.

1.2.1 Basic principles of fluorescence lifetime

Fluorescence lifetime is an essential property that characterizes a fluorescent protein. As illustrated in Fig. 1-2A, after a fluorophore absorbs energy and is excited, electrons transit from the ground state to an excited state, and then gradually releasing energy through radiative or nonradiative pathways, returning to the ground state. Fluorescence lifetime describes the average time that the fluorophore remains in the excited state²⁸.

For an individual excited fluorophore, photon emission is a random event with a defined probability. For a population of the same fluorophores, the probability of photon emission produces a decay in fluorescence intensity after excitation, which can be approximated by an exponential function. In the following equation:

$$F(t) = a_0 e^{-t/\tau} \quad (1 - 1)$$

F is the fluorescence intensity at time t , a_0 is the maximum fluorescence intensity, and τ is the time constant of the mono-exponential decay, corresponding to the time required for fluorescence intensity to decrease to $1/e$ of its maximum value. It represents the mean residence time of the fluorophores in the excited state, which is used to define the fluorescence lifetime of a group of identical fluorophores²⁹.

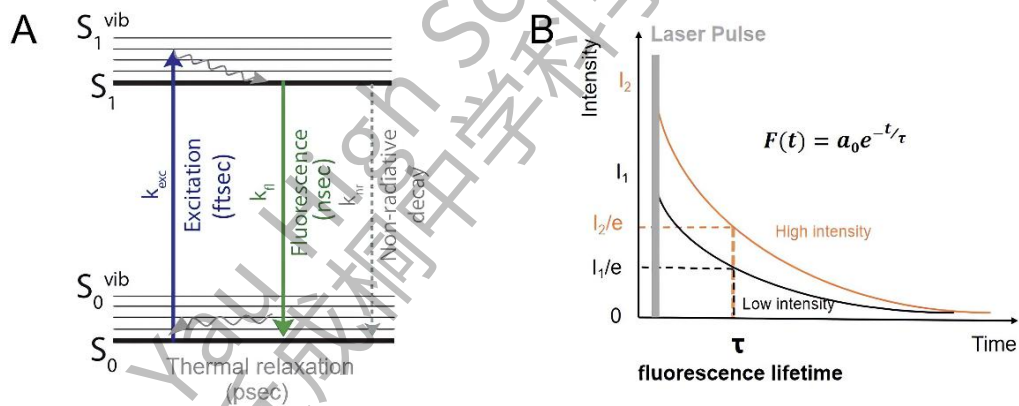


Figure 1-2. Characterization of fluorescence lifetime. (A) Jablonski diagram illustrating excitation and photon emission. (B) Fluorescence intensity decay following excitation by a single light pulse.

The fluorescence lifetimes measured before and after PKA activation are named τ_{pre} and τ_{post} , respectively. The lifetime change $\Delta\tau$ is calculated as:

$$\Delta\tau = \tau_{pre} - \tau_{post} \quad (1 - 2)$$

It reflects the phosphorylation-induced response of the biosensor and serves as a readout of PKA activity: a higher PKA activation level yields a greater number of

phosphorylated biosensors, thus leading to a greater lifetime change.

1.2.2 PKA biosensors based on fluorescence lifetime imaging

Chen et al. introduced FLIM-AKAR in 2014 by combining mEGFP with a dark-YFP acceptor, allowing PKA activity to be monitored through 2-photon FLIM measurements in thick brain tissue³⁰. In 2018, Lei Ma and colleagues developed tAKAR α using a microtubule-anchoring strategy, with a dynamic range of approximately 0.5 ns, and successfully tracked single-cell PKA dynamics in awake mice³¹.

Overall, although PKA biosensors based on different principles have achieved substantial progress, intracellular PKA biosensors based on fluorescence lifetime imaging still suffer from small lifetime changes and limitations in quantitative measurements in living systems. Applying FLIM to intracellular PKA activity therefore requires new reporter domains capable of amplifying lifetime responses.

1.2.3 Screening methods for fluorescence-lifetime biosensors

FLIM is used to measure fluorescence lifetime. The two major approaches are frequency domain and time domain methods.

In frequency domain FLIM, fluorophores are excited periodically with sinusoidally modulated light, and fluorescence lifetime is calculated from the phase delay and amplitude change of emitted photons relative to the excitation light. This approach is suitable for rapid dynamic monitoring but can be sensitive to background noise³².

In time domain FLIM, fluorophores are repeatedly excited with short pulses at a defined frequency. The time interval between excitation and photon emission is recorded and statistically analyzed to generate a fluorescence-decay curve, which is fitted with an exponential function to obtain lifetime parameters. This is known as time-correlated single-photon counting (TCSPC)^{32,33}. TCSPC is considered a gold-standard time-domain fluorescence-lifetime technique. Because the acquisition rate is limited by laser scanning, hundreds of thousands of photons may need to be accumulated to fit the decay curve accurately, resulting in imaging speeds typically below 10 frames. Its low throughput makes it difficult to image rapid dynamics in living neurons and places stringent demands on instrumentation. Therefore, TCSPC is not well suited to the high-throughput strategy established in this study, which expresses biosensor mutants in mammalian cells in 96-well plates and screens them using a high-content platform.

In summary, existing screening systems for fluorescence-lifetime biosensors do not satisfy the requirements of this study. A new high-throughput screening system is thus needed to support fluorescence-lifetime biosensor development.

2 Experimental Methods

2.1 Molecular cloning

In total, we obtained 108 candidates of the target sequence (further elaboration will be made in part 3). The sequences were amplified using Polymerase Chain Reaction (PCR) and inserted into vectors using Gibson Assembly. The vector is then transformed into competent cells and incubated overnight for expression. Colonies were picked for cloning and submitted for sequencing to verify successful plasmid construction. Detailed procedures are listed as follows:

(1) Primer design. The primers designed in this study were divided into short and long primers. Short primers contained 18-30 bp sequences complementary to one end of the target fragment, with an annealing temperature of 58-68°C. Meanwhile, long primers additionally included 25-30 bp overlap region for Gibson assembly.

(2) PCR amplification. All DNA fragments used in this study were obtained by PCR amplification. The template encoding FHA1 and PKAsub was provided by the Jin Zhang laboratory, and the template encoding Venus was provided by the Yulong Li laboratory.

(3) Vector digestion. Two kinds of vectors were used in this study: the pXG2 vector, which was double-digested with *SacI* and *EcoRI*, and the pDisplay vector, which was double-digested with *EcoRI* and *Sall*.

(4) DNA electrophoresis and gel extraction. Electrophoresis of the PCR and digestion products were performed. Afterwards, DNA fragments were purified by gel extraction.

(5) Gibson assembly. The target sequence is inserted into the vector. The Gibson mix was prepared in the laboratory based on a published formulation and optimized by replacing PEG8000 with 1,2-propanediol. Multiple enzymes act synergistically: a 5' exonuclease digests DNA ends to generate single-stranded homologous regions; complementary fragments anneal to form double-stranded DNA; DNA polymerase fills the gaps; and ligase seals the phosphodiester backbone. The reaction was performed at 50°C for 1 h, followed by inactivation at 80°C for 5 min.

(6) Transformation of competent cells. Gibson assembly products were transformed into competent cells. Cells were plated on LB agar containing ampicillin and cultured overnight at 37°C. Colonies were picked for cloning and submitted for sequencing to verify successful plasmid construction.

2.2 Bacterial expression and lysis

A correctly sequenced pXG2 plasmid containing the desired gene sequence was transformed into 50 µL DH5α competent cells. The bacterial suspension was plated on LB agar containing ampicillin and incubated upside down overnight at 37°C. Individual colonies were then streaked with approximately 5-10 mm spacing and incubated at 37°C for 14-16 h. Afterward, the cultures were kept at room temperature for approximately 56-58 h, giving a total expression time of 72 h.

Following expression, bacterial pellets were resuspended in PBS and lysed by sonication. The resulting lysates were centrifuged, and the supernatant was collected and filtered through a 0.22 µm syringe filter to remove insoluble material.

2.3 Spectral measurements

Chemical dyes were incubated with bacterial lysates containing the self-labeling proteins under dark, cold conditions. The mixtures were then purified using spin chromatography columns to remove unbound dyes and exchanged into PBS. The purified protein samples were transferred to a 384-well plate, with three technical replicates for each condition. Single-photon emission spectra were subsequently measured using a microplate reader.

2.4 HEK 293T cell culture, transfection, and staining

HEK 293T cells were cultured in complete DMEM at 37°C with 5% CO₂ and passaged approximately every two days according to cell growth. Purified pDisplay plasmids were transfected into the cells using polyethyleneimine (PEI) at 70-80% confluence at the ratio of PEI:DNA = 3:1. After transfection, the medium was replaced with fresh complete medium, and cells were incubated for 24-36 h. For labeling, cells were incubated with dye425-HTL or SiR650-TMP for 3 h, followed by replacement with fresh complete medium and further incubation before imaging.

2.5 High-content live-cell imaging and analysis

Before imaging, the medium in the 96-well plate was replaced with Tyrode's solution while the pH was adjusted to 7.35-7.45.

HEK 293T cells were imaged using an Operetta CLS high-content live-cell

imaging and analysis system with a 20× water-immersion objective (NA 1.0). For the FRET system, the Donor channel used 355-385 nm excitation and 430-500 nm emission filters, recording F_D ; the Acceptor channel used 355-385 nm excitation and 515-580 nm emission filters, recording F_A ; the Venus channel used 460-490 nm excitation and 500-550 nm emission filters, recording F_{Venus} ; and the Reference channel used 615-645 nm excitation and 655-760 nm emission filters, recording F_R . Nine fields of view were selected for analysis in each well. Signals were analyzed using formulas configured in the high-content analysis software. Data were analyzed according to the following formulas:

$$\text{Donor response: } \frac{\Delta D}{D_0} = \frac{F_{D_{post}} - F_{D_{pre}}}{F_{D_{pre}}} \quad (2 - 1)$$

$$\text{Acceptor response: } \frac{\Delta A}{A_0} = \frac{F_{A_{post}} - F_{A_{pre}}}{F_{A_{pre}}} \quad (2 - 2)$$

$$\text{Post - treatment brightness} = \frac{F_{A_{post}}}{F_{D_{post}}} \quad (2 - 3)$$

$$\text{Index} = \frac{\left| \frac{\Delta A}{A_0} - \frac{\Delta D}{D_0} \right|}{\frac{\frac{\Delta A}{A_0}}{\left| \frac{\Delta A}{A_0} \right|}} \quad (2 - 4)$$

2.6 Fluorescence lifetime imaging and analysis

Before FLIM, the medium in the 96-well plate was likewise replaced with Tyrode's solution. FLIM was performed using a Leica SP8 STED inverted confocal microscope with a 20× objective on HEK 293T cells expressing the biosensors. Venus was excited at 514 nm and fluorescence was collected from 525-575 nm. Images were acquired at 1024 × 1024 pixels, with each image representing eight consecutive acquisitions accumulated to collect sufficient photons. More than 100,000 photons per field of view were required for analysis.

In this research, SEM stands for standard error of the mean.

3 Experimental Principle

To address the technical challenge of detecting and quantitatively evaluating PKA activity in living cells with high spatiotemporal resolution, this study combined protein engineering with high-throughput screening to develop a genetically encoded PKA biosensor based on fluorescence lifetime imaging and to validate its performance in cells.

3.1 Molecular design of FLIM-PKA

Fluorescent-protein-based biosensors generally contain two functional domains: a sensing domain and a reporter domain. The sensing domain typically poses sensitivity and specificity toward the target, either participating directly in the signaling pathway of interest or is constructed based on proteins involved in that pathway. The sensing domain framework of ExRai-AKAR2 developed by the Jin Zhang laboratory was adopted²⁷ in this study. A PKA substrate peptide (PKAsub) and the phosphopeptide-binding domain FHA1 are used to construct a phosphorylation-dependent molecular switch for dynamic monitoring of PKA activity. The amino acid sequence of PKAsub is LRRATLVD, with T serving as the phosphorylation site.

The fluorescent protein in the reporter domain determines the brightness and excitation/emission wavelengths of the biosensor, while its interaction with the sensing domain influences dynamic range and sensitivity. This study prioritizes fluorescent proteins with tunable fluorescence quantum yields. The blue-green fluorescent protein mTurquoise2 (excitation/emission: 434/474 nm), although used for calcium biosensors, has short-wavelength characteristics that result in poor tissue penetration and lower signal-to-noise ratios. By comparison, the yellow fluorescent protein Venus (excitation/emission: 515/528 nm) has more favorable spectral characteristics. Also, the glutamate biosensor iGluSnFR3.v857 derived from it shows a marked increase in quantum yield upon substrate binding (0.26 to 0.93), making Venus a favorable candidate for developing a PKA fluorescence-lifetime biosensor. Furthermore, to improve the transmission efficiency of conformational changes, Venus was circularly permuted between L141 and E142. Upon phosphorylation or dephosphorylation of the substrate peptide, conformational changes in the β -barrel of cpVenus can thereby be efficiently converted into fluorescence changes.

Thus, the PKA biosensor designed in this study consists of a sensing domain composed of PKAsub and FHA1, a reporter domain using cpVenus, and linker peptides connecting the domains. Its mechanism is illustrated in Fig. 3-1A and B.

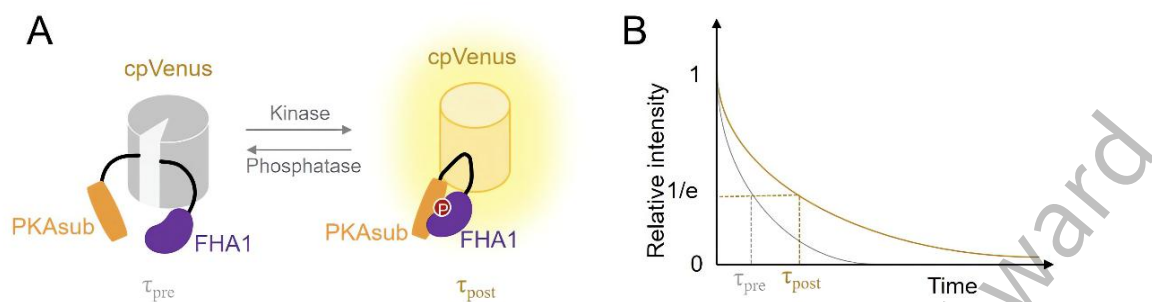


Figure 3-1. Principle of the PKA biosensor based on fluorescence lifetime imaging. (A) Molecular-switch mechanism. (B) Fluorescence-lifetime reporting mechanism.

When PKA activity is low, PKA_{sub} is not phosphorylated and cpVenus has a fluorescence lifetime of τ_{pre} . When PKA activity is high, PKA phosphorylates PKA_{sub} while FHA1 binds to the phosphate group, inducing a conformational change in the sensing domain that is transmitted to cpVenus and alters its fluorescence lifetime to τ_{post} . By measuring the change in cpVenus fluorescence lifetime before and after phosphorylation, the lifetime change can be quantitatively converted into a change in PKA activity.

3.2 Design of high-throughput screening strategy

As discussed above, FLIM-based screening is constrained by imaging speed and instrumentation and therefore has limited throughput. The relatively slow lifetime measurement can potentially be converted into an easier-to-measure parameter, such as fluorescence intensity.

$$\tau \propto \varphi \quad (3-1)$$

From this formula, we can see that fluorescence lifetime τ is proportional to quantum yield φ , which describes how efficiently a fluorescent-protein chromophore converts absorbed energy into emitted photons.

Meanwhile, according to the Beer-Lambert law:

$$F \propto \varphi \cdot I_{abs} \quad (3-2)$$

$$I_{abs} \approx I_0 \cdot \epsilon c l \ln 10 \quad (3-3)$$

where F is fluorescence intensity, φ is quantum yield, I_0 is excitation-light intensity, I_{abs} is absorbed-light intensity, ϵ is the extinction coefficient, c is fluorophore concentration, and l is the optical path length. The extinction coefficient ϵ describes the efficiency with which a fluorescent-protein chromophore absorbs light at a

specific wavelength.

Under fixed experimental conditions, i.e., when I_0 , c , and l remain constant:

$$F \propto \varphi \cdot \varepsilon \quad (3-4)$$

Therefore, fluorescence intensity F is proportional to the quantum yield φ and extinction coefficient ε .

Combining Eq.3-1 and 3-4, a suitable strategy is required to distinguish changes in quantum yield from changes in extinction coefficient. The desired biosensor should show fluorescence-intensity changes primarily caused by quantum yield changes rather than extinction-coefficient changes, because the former are directly related to fluorescence lifetime.

This research followed the FRET system designed by Lars Hellweg and colleagues³⁴. To implement this strategy, HaloTag, a self-labeling protein, was linked to cpVenus through a flexible peptide and labeled with a fluorescent ligand, serving as a FRET donor that emits stable energy under fixed excitation conditions. This converts changes in the extinction coefficient and quantum yield of cpVenus that is caused by conformational changes into measurable changes in emission intensity of the donor and cpVenus, as shown in Figure 3-2.

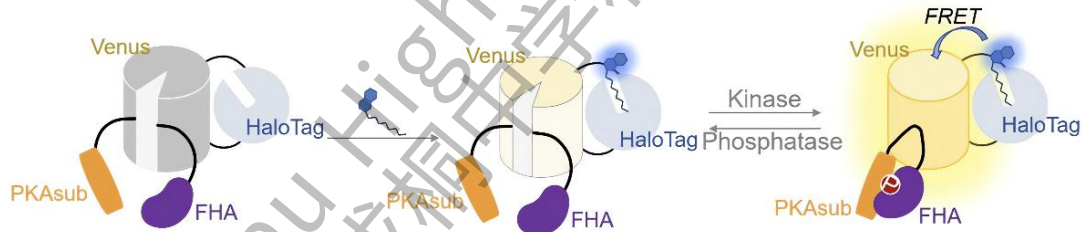


Figure 3-2. Principle of the PKA biosensor based on a FRET system.

After the chemical dye labels HaloTag, the dye-HaloTag complex serves as a stable energy donor. When PKA activity is low, PKAsub is not phosphorylated; the fluorescence intensity of the chemical-dye and the cpVenus are FD_{pre} and FA_{pre} , respectively. When PKA activity is high, PKA phosphorylates PKAsub, FHA1 binds the phosphate group, and the resulting conformational change is transmitted to cpVenus. The corresponding fluorescence intensities are FD_{post} and FA_{post} .

If the extinction coefficient increases while quantum yield remains unchanged, as shown in Fig. 3-3A, cpVenus absorbs donor energy more efficiently and captures more energy. Consequently, donor fluorescence decreases because more energy is

transferred, whereas cpVenus fluorescence increases because the absorbed energy increases. If quantum yield increases while extinction coefficient remains unchanged, which is the ideal case in this study, cpVenus converts absorbed energy into photons more efficiently, increasing cpVenus fluorescence. However, because the amount of energy transferred does not change, donor fluorescence remains unaffected, as shown in Fig. 3-3B.

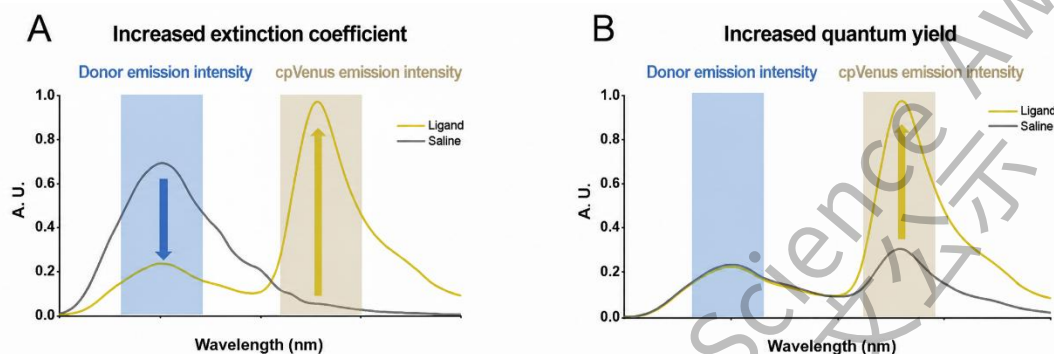


Figure 3-3. Principle of distinguishing changes in fluorescent-protein extinction coefficient and quantum yield using the FRET system.

To construct this FRET system, an appropriate dye had to be selected for HaloTag. After HaloTag was labeled, the emission spectrum of the dye needed sufficient overlap with the excitation spectrum of cpVenus to enable effective energy transfer. At the same time, the emission spectrum of the dye and the emission spectrum of cpVenus needed sufficient separation to allow independent measurement of each fluorophore. The chemical dye dye425 developed by the Luke D. Lavis laboratory was selected and conjugated to a HaloTag ligand (HTL), generating dye425-HTL, which selectively binds HaloTag and forms a stable covalent complex.

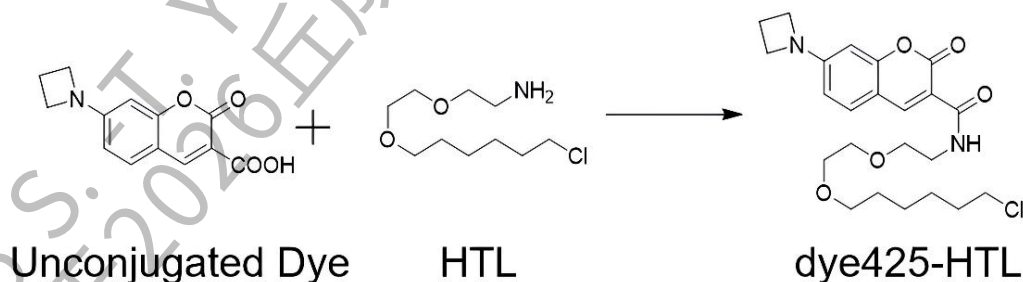


Figure 3-4. Chemical structure of dye425 used in the FRET system.

3.3 Linker peptide optimization

The amino acid peptide connecting the sensing and reporter domains is referred

to as the linker peptide. As an artificial short peptide, the linker serves dual functions in assisting fluorescent-protein folding and transmitting conformational signals. Its amino acid composition and length strongly affect the dynamic range of the biosensor. In this study, linker truncation was used to optimize linker length. To avoid disrupting key interactions, truncations were introduced from the central region of the linker, i.e., outward from the junction between the sensing and reporter domains. As shown in Fig. 3-5, linker1 had 12 possible truncation patterns and linker2 had 9, yielding $12 \times 9 = 108$ combinations.

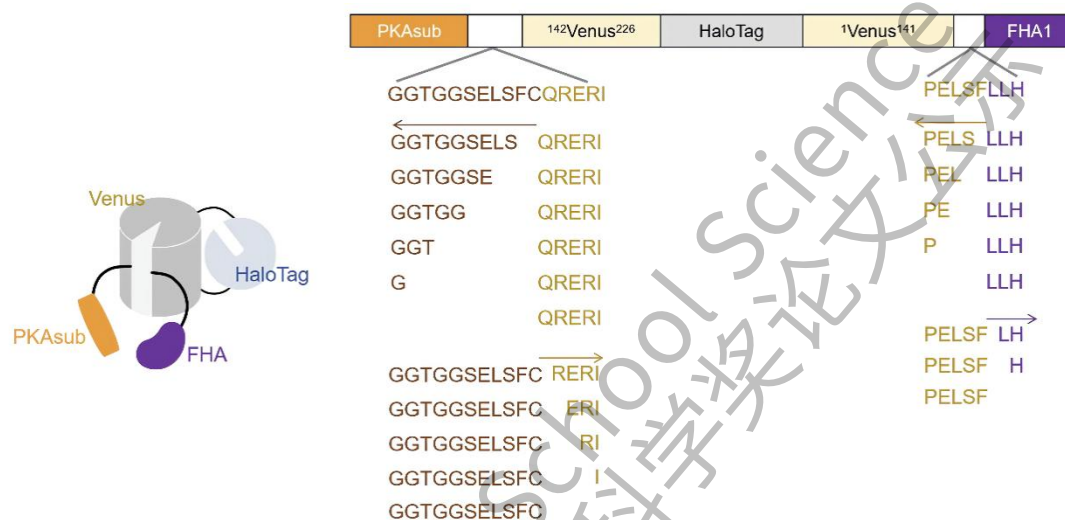


Figure 3-5. Design of linker truncations for FRET-PKA.

4 Results and Analysis

4.1 Validation of the high-throughput screening strategy

4.1.1 Spectral measurements

To ensure that the screening system could support FRET and to guide selection of filters for subsequent experiments using the Operetta CLS high-content system, the spectral properties of dye425-HTL and Venus were first measured using a Tecan full-wavelength multifunctional microplate reader.

As shown in Fig. 4-1A, dye425-HTL, after covalent conjugation to HaloTag, exhibited an excitation maximum at 425 nm and an emission maximum at 484 nm. Venus exhibited an excitation maximum at 517 nm and an emission maximum at 530 nm, consistent with Venus spectral data in the FPbase database and supporting the reliability of the measurements. To assess the possibility of FRET between dye425-

HTL and Venus, their emission and excitation spectra were plotted on the same coordinate system. As shown in Fig. 4-1B, the dye emission spectrum and the Venus excitation spectrum overlapped more than 30%, providing theoretical support for FRET. The emission maxima were also sufficiently separated to permit simultaneous monitoring of donor and acceptor fluorescence.

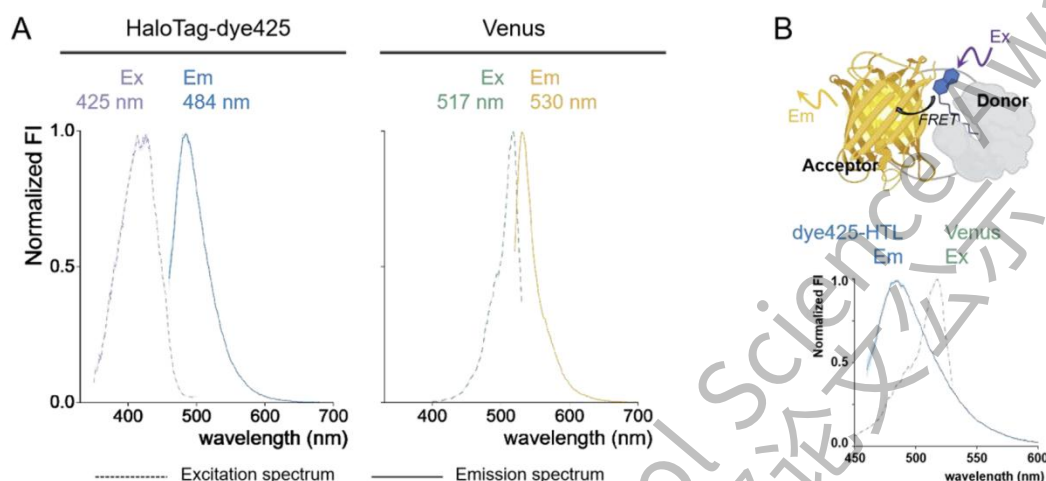


Figure 4-1. Spectra of HaloTag-dye425 and Venus.

(A) Spectra of dye425-HTL and Venus. Dashed lines indicate excitation spectra and solid lines indicate emission spectra; $n=3$ wells, shaded regions represent SEM.

(B) Rational analysis of FRET between dye425-HTL and Venus.

4.1.2 Establishment of the FRET system in HEK 293T cells

To comprehensively evaluate the applicability of the FRET system and establish a cellular basis for subsequent biosensor screening, FRET was further validated in HEK 293T cells.

Three constructs were expressed: HaloTag alone and Venus alone as controls, and Venus-HaloTag as the FRET system. All three constructs were linked to IRES-TMP expression reference, in which the internal ribosome entry site (IRES) enables translation of the downstream TMP-tag. The TMP-tag itself is a non-fluorescent protein but can be labeled with a TMP-conjugated dye. Signals were collected using the Operetta CLS high-content live-cell imaging system with four channels: Channel 1 (Donor), 355-385 nm excitation and 430-500 nm emission, to monitor changes in chemical-dye fluorescence; Channel 2 (Acceptor), 355-385 nm excitation and 515-580 nm emission, to detect Venus emission generated by FRET; Channel 3 (Venus), 460-490 nm excitation and 500-550 nm emission, to independently verify Venus expression and facilitate comparison between fluorescence-intensity and

fluorescence-lifetime changes in subsequent screening; and Channel 4 (Reference), 615-645 nm excitation and 655-760 nm emission, using SiR650-TMP, a far-red fluorescent TMP ligand used to label the TMP-tag. Donor and acceptor fluorescence intensities were separately divided by the SiR650-TMP reference intensity to obtain ratios, which were used to reduce intensity variations caused by cellular autofluorescence and background interference.

Cells were stained 24 h after transfection. After 3 h staining, the medium was replaced and cells were incubated for at least another 2 h before imaging.

At comparable protein expression levels in HEK 293T cells, signals from the systems were analyzed in each channel. In the Donor channel, fluorescence was observed only in successfully transfected cells with effective chemical-dye labeling. Except for the Venus-only control, the other two systems exhibited clear donor signals, demonstrating negligible cross-excitation or bleed-through of Venus into the Donor channel. The HaloTag-only system was substantially brighter than the FRET system, indicating that FRET quenched dye fluorescence as energy was transferred to Venus. The Acceptor channel showed weak signals in both HaloTag-only and Venus-only controls, indicating minor bleed-through, but the FRET system produced substantially stronger signals. This interference can be reduced computationally in subsequent experiments while preserving the FRET-specific signal. As shown in Figure 4-2B, the FRET ratio was 0.26 ± 0.02 for HaloTag alone and 2.57 ± 0.01 for Venus-HaloTag, further validating the successful establishment of FRET between the donor and Venus.

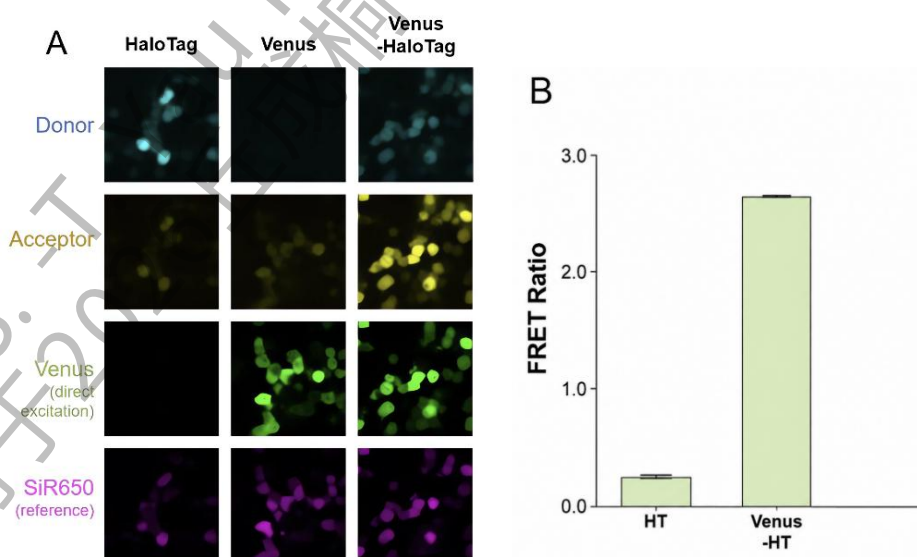


Figure 4-2. Signal analysis of the FRET systems in HEK 293T cells.

(A) Representative images in Donor, Acceptor, Venus, and SiR650 channels. Scale bar, 50 μm . (B) FRET ratio bar graph of the FRET system. $n = 3$ wells; error bars represent SEM.

4.2 Linker peptide optimization results

Molecular cloning generated 97 reliable biosensor plasmid candidates, which were purified and individually transfected into HEK 293T cells. Cells were labeled with chemical dye 24 hours after transfection. The 11 unsuccessful constructs were caused by unsuccessful establishment of plasmids or fluorescence intensity being too low.

Forskolin (Fsk) is a potent adenylyl cyclase activator that induces intracellular cAMP production, and increased cAMP promotes PKA activation. IBMX is a broad-spectrum phosphodiesterase (PDE) inhibitor that prevents cAMP degradation, thereby increasing intracellular cAMP levels while sustaining PKA activation and substrate phosphorylation, stabilizing the readout of PKA activity. Biosensor responses were measured after incubation with 50 μM Fsk and 100 μM IBMX for 20 min using the Index response amplitude.

Four channels were recorded as described above: Channel 1 (Donor), 355-385 nm excitation and 430-500 nm emission, to monitor changes in chemical-dye emission before and after drug stimulation; Channel 2 (Acceptor), 355-385 nm excitation and 515-580 nm emission, to monitor drug-induced changes in Venus emission through FRET; Channel 3 (Venus), 460-490 nm excitation and 500-550 nm emission, to independently assess Venus expression and compare changes in fluorescence intensity with fluorescence lifetime; and Channel 4 (Reference), 615-645 nm excitation and 655-760 nm emission, using the covalent complex of SiR650-TMP and the self-labeling protein TMP as the reference to reduce autofluorescence and background interference.

Candidates were initially ranked according to Index response amplitude. Index response measures the difference between acceptor and donor responses; its magnitude reflects the extent of this difference, while its sign is determined by the sign of the acceptor response. As the desirable candidates should display a larger difference between acceptor and donor response, the 5 candidates with the largest and the 20 candidates with the smallest Index values were selected from the 97 candidates, as shown in Fig. 4-3A. These 25 candidates were subjected to replicate experiments with $n=3$ wells, as shown in Fig. 4-3B. The candidate with an 8-residue N-terminal truncation of linker1 and no truncation of linker2 (N Δ 8, C Δ 0) showed an Index

response of approximately -0.35 and post-treatment brightness of approximately 0.35 in the primary screen. In the replicate experiment, it showed an Index response of -0.17 ± 0.02 and post-treatment brightness of 0.47 ± 0.01 .

Based on the replicate results, six candidates were selected for fluorescence-lifetime imaging. Consistent with expectations, N Δ 8/C Δ 0 produced the largest lifetime change, -0.75 ± 0.05 ns ($n=3$ wells), and was named FRET-PKA.

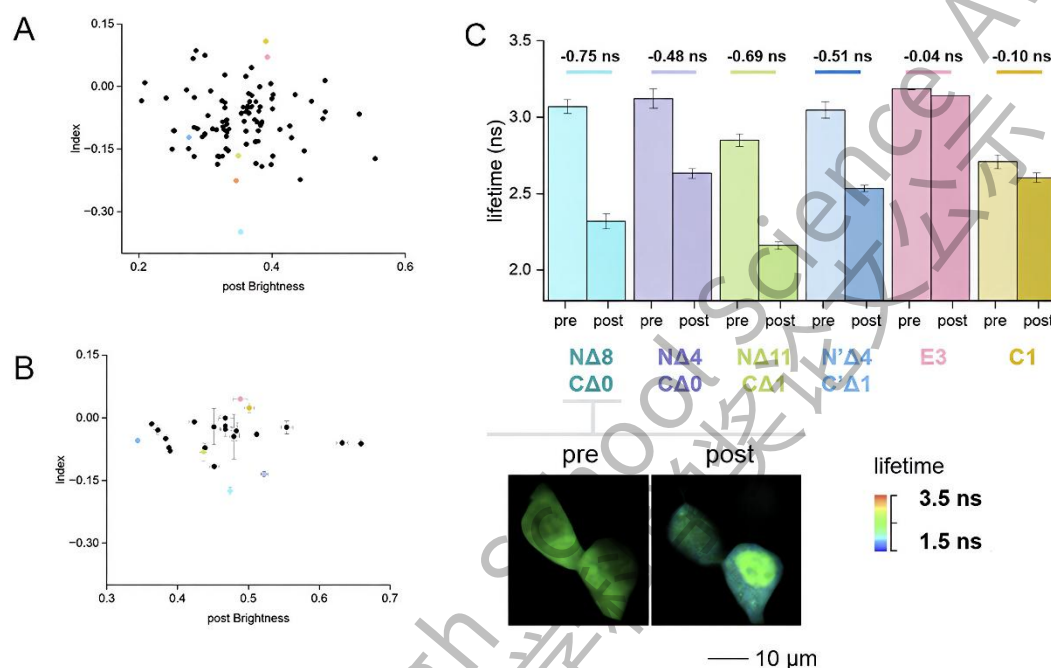


Figure 4-3. Screening results for FRET-PKA linker truncations.

(A) Primary screening. (B) Replicate screening. (C) Fluorescence-lifetime responses. Lifetime scale: 1.5-3.5 ns; cell scale bar: 10 μ m. Post-treatment data were obtained after 20 min incubation with 50 μ M Fsk and 100 μ M IBMX. $n = 3$ wells; error bars represent SEM.

4.3 Pharmacological experiments

The FRET system composed of a chemical dye and fluorescent protein was used for high-throughput screening of candidate biosensors suitable for FLIM. The central goal of the study was to develop a single-fluorescent-protein FLIM-PKA biosensor with a high dynamic range and quantitative readout. Thus, after identifying FRET-PKA as a promising candidate, the HaloTag sequence was removed while retaining the sensing and reporter domains, yielding the final FLIM-PKA biosensor.

Pharmacological experiments were performed to systematically validate the

dynamic performance of FRET-PKA and FLIM-PKA. The two biosensor plasmids were first transfected into HEK 293T cells. H89, a PKA inhibitor, was applied at 10 μ M to suppress basal PKA activity, and the initial fluorescence lifetime was measured as the baseline. Next, 50 μ M Fsk and 100 μ M IBMX were added to activate the PKA pathway, and fluorescence-lifetime changes were measured after 20 min. Finally, 50 μ M H89 was added again and incubated for 20 min to assess lifetime recovery and evaluate signal reversibility and selectivity.

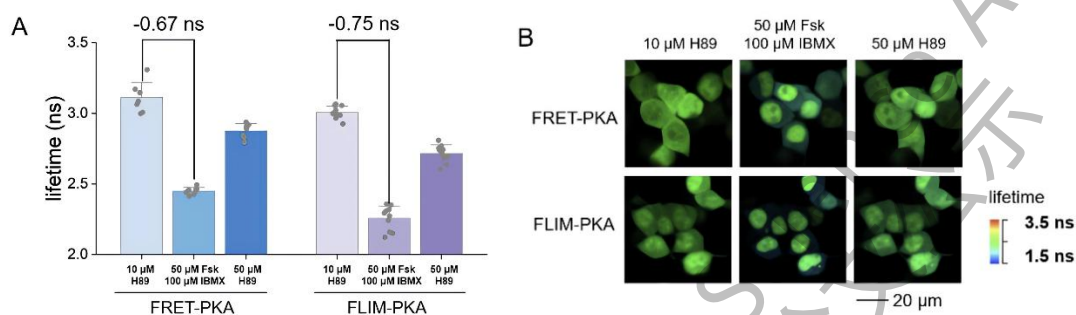


Figure 4-4. Fluorescence-lifetime responses of FRET-PKA and FLIM-PKA.

(A) Lifetime responses; post-treatment data were collected after 20 min drug incubation. Error bars represent SEM.

(B) Representative cellular FLIM images; lifetime scale: 1.5-3.5 ns; scale bar: 20 μ m.

As shown in Fig. 4-4, FRET-PKA showed a fluorescence-lifetime change of -0.67 ± 0.05 ns ($n=7$ cells), whereas FLIM-PKA showed a change of -0.75 ± 0.04 ns ($n=10$ cells), both substantially greater than those reported for tAKAR α (-0.5 ns) and FLIM-AKAR (-0.2 ns). After addition of 50 μ M H89, the lifetime recovered by 0.43 ± 0.03 ns for FRET-PKA (64.2% recovery) and 0.46 ± 0.05 ns for FLIM-PKA (61.3% recovery), demonstrating partial reversibility and relatively high selectivity of biosensors.

Further analysis showed that the response amplitudes of FRET-PKA and FLIM-PKA were highly consistent, indicating that introduction of HaloTag did not substantially affect cpVenus conformational changes or protein folding. This property establishes a reliable framework for efficient FLIM-biosensor screening based on the FRET strategy.

5 Discussion and Perspectives

5.1 Biosensor Performance

The FLIM-PKA biosensor developed in this study exhibited a fluorescence-lifetime change of -0.75 ± 0.04 ns following activation of the PKA pathway. This response is larger than the lifetime changes reported for previously developed fluorescence-lifetime-based PKA biosensors. FLIM-AKAR, which uses an mEGFP/dark-YFP donor-acceptor pair, exhibits a lifetime response of approximately 0.2 ns³⁰, whereas tAKAR α , developed for two-photon fluorescence lifetime imaging of PKA activity in neurons and awake mice, achieves a dynamic range of approximately 0.5 ns³¹. In comparison, the response amplitude of FLIM-PKA in this study was approximately 1.5-fold that of tAKAR α and 3.75-fold that of FLIM-AKAR.

The increased lifetime response is important for quantitative PKA imaging because a larger separation between the pre- and post-activation fluorescence lifetimes may improve the ability to detect relatively small changes in kinase activity.

5.2 Screening Strategy

A major challenge in developing FLIM-based biosensors is the relatively low throughput of direct fluorescence-lifetime measurements. TCSPC requires the accumulation of large numbers of photons for reliable lifetime determination, making direct screening of large mutant libraries inefficient.

To address this limitation, this study established a FRET-based strategy by connecting a self-labeling protein HaloTag to the sensor, which enabled candidate biosensors to be initially evaluated through high-content fluorescence-intensity imaging. Combining such strategy with subsequent FLIM validation, N Δ 8/C Δ 0, showing the largest lifetime change, was successfully selected among the 97 candidates. Importantly, FRET-PKA and the HaloTag-free FLIM-PKA exhibited similar lifetime responses of -0.67 ± 0.05 ns and -0.75 ± 0.04 ns, respectively. This agreement supports the feasibility of using the FRET system to identify promising candidates while reserving direct FLIM measurements for final validation.

5.3 Innovations and Contributions

The major innovations of this study can be summarized in two aspects:

(1) Development of a single-fluorophore FLIM-based PKA biosensor. FLIM-PKA combines the design strategy of a single fluorescent-protein with fluorescence-lifetime readout, distinguishing it from previous single-fluorophore biosensors based on intensity measurements and previous FLIM-based biosensors utilizing donor-acceptor pairs. This design retains the quantitative advantages of fluorescence-lifetime

imaging, including excluding unwanted interfering factors of sensor density, expression levels, and tissue scattering, while also reducing the complexity and size associated with systems containing two fluorophores, providing advantages for future *in vivo* applications.

(2) Development of high throughput screening methods. To overcome the setbacks of FLIM, a FRET system was created based on energy transfer between a donor and a fluorescent-protein, to systematically convert fluorescence-lifetime into fluorescence-intensity. This strategy provides an efficient approach for optimizing fluorescence-lifetime biosensors.

5.4 Limitations and Perspectives

First, FLIM-PKA has currently been characterized primarily in HEK 293T cells, and whether its lifetime response can be maintained in neurons and *in vivo* remains to be determined. Further work should therefore focus on characterizing properties of the sensor in cultured neurons and performing *in vivo* trials on mice.

In addition, FLIM-PKA has so far undergone only linker truncation. Further modification of linker sequence and cpVenus may provide additional improvements.

Lastly, the successful establishment of a molecular system for kinase activity measurement isn't limited to PKA. By adapting the substrate sequence, this system has the potential to contribute to the monitoring of other kinase activities. Biosensors for kinases such as CaMKII and protein kinase C (PKC) could potentially be created based on the design in this study.

References

1. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The Protein Kinase Complement of the Human Genome. *Science*. 2002;298(5600):1912-1934. doi:10.1126/science.1075762
2. Klebl B, Müller G, Hamacher M, Mannhold R, Kubinyi H, Folkers G. *Protein Kinases as Drug Targets*. Wiley-VCH Weinheim; 2011.
3. López-Bigas N, Ouzounis CA. Genome-wide identification of genes likely to be involved in human genetic disease. *Nucleic Acids Res*. 2004;32(10):3108-3114. doi:10.1093/nar/gkh605
4. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304(5676):1497-1500. doi:10.1126/science.1099314
5. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007;448(7153):561-566. doi:10.1038/nature05945
6. Kluss JH, Beilina A, Williamson CD, Lewis PA, Cookson MR, Bonet-Ponce L. Lysosomal positioning regulates Rab10 phosphorylation at LRRK2+ lysosomes. *Proc Natl Acad Sci U S A*. 2022;119(43):e2205492119. doi:10.1073/pnas.2205492119
7. Attwood MM, Fabbro D, Sokolov AV, Knapp S, Schiöth HB. Trends in kinase drug discovery: targets, indications and inhibitor design. *Nat Rev Drug Discov*. 2021;20(11):839-861. doi:10.1038/s41573-021-00252-y
8. Walsh DA, Perkins JP, Krebs EG. An adenosine 3', 5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. *Journal of Biological Chemistry*. 1968;243(13):3763-3765.
9. Fernandes HB, Riordan S, Nomura T, et al. Epac2 Mediates cAMP-Dependent Potentiation of Neurotransmission in the Hippocampus. *J Neurosci*. 2015;35(16):6544-6553. doi:10.1523/JNEUROSCI.0314-14.2015
10. Lee K, Kobayashi Y, Seo H, et al. Involvement of cAMP-guanine nucleotide exchange factor II in hippocampal long-term depression and behavioral flexibility. *Mol Brain*. 2015;8(1):38. doi:10.1186/s13041-015-0130-1
11. Bourtchouladze R, Patterson SL, Kelly MP, Kreibich A, Kandel ER, Abel T. Chronically increased G_s α signaling disrupts associative and spatial learning. *Learn*

Mem. 2006;13(6):745-752. doi:10.1101/lm.354106

12. Kelley D, Bhattacharyya A, Lahvis G, Yin J, Malter J, Davidson R. The cyclic AMP phenotype of fragile X and autism. *Neuroscience & Biobehavioral Reviews*. 2008;32(8):1533-1543. doi:10.1016/j.neubiorev.2008.06.005

13. Neer EJ, Clapham DE. Roles of G protein subunits in transmembrane signalling. *Nature*. 1988;333(6169):129-134. doi:10.1038/333129a0

14. Janetopoulos C, Jin T, Devreotes P. Receptor-Mediated Activation of Heterotrimeric G-Proteins in Living Cells. *Science*. 2001;291(5512):2408-2411. doi:10.1126/science.1055835

15. Bünemann M, Frank M, Lohse MJ. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc Natl Acad Sci USA*. 2003;100(26):16077-16082. doi:10.1073/pnas.2536719100

16. Uhler MD, Chrivia JC, McKnight GS. Evidence for a second isoform of the catalytic subunit of cAMP-dependent protein kinase. *J Biol Chem*. 1986;261(33):15360-15363.

17. Uhler MD, McKnight GS. Expression of cDNAs for two isoforms of the catalytic subunit of cAMP-dependent protein kinase. *J Biol Chem*. 1987;262(31):15202-15207.

18. Jahnsen T, Hedin L, Kidd VJ, Schulz T, Richards JS. Molecular cloning of cDNA for a hormone-regulated isoform of the regulatory subunit of type II cAMP-dependent protein kinase from rat ovaries. *Methods Enzymol*. 1988;159:318-324. doi:10.1016/0076-6879(88)59032-8

19. Ma H, Deacon S, Horiuchi K. The challenge of selecting protein kinase assays for lead discovery optimization. *Expert Opin Drug Discov*. 2008;3(6):607-621. doi:10.1517/17460441.3.6.607

20. Kruk M, Widstrom N, Jena S, et al. Assays for tyrosine phosphorylation in human cells. *Methods Enzymol*. 2019;626:375-406. doi:10.1016/bs.mie.2019.06.026

21. Gest AMM, Sahan AZ, Zhong Y, Lin W, Mehta S, Zhang J. Molecular Spies in Action: Genetically Encoded Fluorescent Biosensors Light up Cellular Signals. *Chem Rev*. 2024;124(22):12573-12660. doi:10.1021/acs.chemrev.4c00293

22. Nagai Y, Miyazaki M, Aoki R, et al. A fluorescent indicator for visualizing cAMP-induced phosphorylation in vivo. *Nat Biotechnol*. 2000;18(3):313-316. doi:10.1038/73767

23. Zhang J, Ma Y, Taylor SS, Tsien RY. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc Natl Acad Sci U S A*. 2001;98(26):14997-15002. doi:10.1073/pnas.211566798
24. Zhang J, Hupfeld CJ, Taylor SS, Olefsky JM, Tsien RY. Insulin disrupts beta-adrenergic signalling to protein kinase A in adipocytes. *Nature*. 2005;437(7058):569-573. doi:10.1038/nature04140
25. Allen MD, Zhang J. Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem Biophys Res Commun*. 2006;348(2):716-721. doi:10.1016/j.bbrc.2006.07.136
26. Mehta S, Zhang Y, Roth RH, et al. Single-fluorophore biosensors for sensitive and multiplexed detection of signalling activities. *Nat Cell Biol*. 2018;20(10):1215-1225. doi:10.1038/s41556-018-0200-6
27. Zhang JF, Liu B, Hong I, et al. An ultrasensitive biosensor for high-resolution kinase activity imaging in awake mice. *Nat Chem Biol*. 2021;17(1):39-46. doi:10.1038/s41589-020-00660-y
28. Datta R, Heaster TM, Sharick JT, Gillette AA, Skala MC. Fluorescence lifetime imaging microscopy: fundamentals and advances in instrumentation, analysis, and applications. *J Biomed Opt*. 2020;25(7):1-43. doi:10.1117/1.JBO.25.7.071203
29. Gratton E, Breusegem S, Sutin J, Ruan Q, Barry N. Fluorescence lifetime imaging for the two-photon microscope: time-domain and frequency-domain methods. *J Biomed Opt*. 2003;8(3):381-390. doi:10.1117/1.1586704
30. Chen Y, Saulnier JL, Yellen G, Sabatini BL. A PKA activity sensor for quantitative analysis of endogenous GPCR signaling via 2-photon FRET-FLIM imaging. *Front Pharmacol*. 2014;5:56. doi:10.3389/fphar.2014.00056
31. Ma L, Jongbloets BC, Xiong WH, et al. A Highly Sensitive A-Kinase Activity Reporter for Imaging Neuromodulatory Events in Awake Mice. *Neuron*. 2018;99(4):665-679.e5. doi:10.1016/j.neuron.2018.07.020
32. Lakowicz JR, ed. *Principles of Fluorescence Spectroscopy*. Springer US; 2006. doi:10.1007/978-0-387-46312-4
33. *The Bh TCSPC Handbook 10th Edition*.
34. Hellweg L, Edenhofer A, Barck L, et al. A general method for the development of multicolor biosensors with large dynamic ranges. *Nat Chem Biol*. 2023;19(9):1147-

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The research topic was assigned by the laboratory. During the initial stage of the project, Professor Li provided guidance on the experimental design and participated in discussions that contributed to the development of the overall research strategy. In particular, we jointly considered the molecular design of the biosensor and the strategy for overcoming the low throughput of conventional fluorescence-lifetime-based biosensor screening. Following the establishment of the research plan, I independently carried out the experimental work, including molecular cloning, protein expression and spectral characterization, cell culture and transfection, high-content live-cell imaging, fluorescence lifetime imaging, pharmacological experiments, data acquisition and processing, and imaging analysis. These procedures constituted the major experimental components of this study.

Although no major difficulties interrupted the progress of the project, the optimization and screening process required careful comparison among a large number of candidates and validation of the relationship between the high-throughput screening results and direct fluorescence-lifetime measurements. This process required me to evaluate experimental results critically and determine which candidates should proceed to further validation rather than relying on a single measurement. Through repeated screening and FLIM validation, I gained a deeper understanding of how experimental evidence can be used to evaluate and refine an initial research design.

I independently completed the first draft of this essay after finishing the experimental work and data analysis. I am grateful to Professor Yulong Li for reviewing and providing suggestions that helped me improve its scientific presentation. I would also like to thank my school instructor, Yongyun Gang, who reviewed parts of the essay after the completion of my first draft and provided

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