Molecular Biosensors and Intracellular Molecular Imaging Postprint

Authors: Wang Dianbing, Cui Zongqiang, Zhang Xianen

Date: 2018-01-09T00:00:00+00:00

Abstract

Molecular biosensors are sensors constructed from biological macromolecules via genetic recombination or DNA synthesis, capable of real-time, visualized detection of key molecular events in living cells and in vivo. Currently, molecular biosensors with high research interest and broad applications include molecular beacons (MB), resonance energy transfer systems (fluorescence resonance energy transfer and bioluminescence resonance energy transfer), and molecular fluorescence complementation systems (such as bimolecular fluorescence complementation and trimolecular fluorescence complementation). This article introduces the principles and characteristics of these molecular biosensors, emphasizing their applications in live-cell molecular imaging, such as investigating intracellular protein-protein interactions, exploring the localization, movement, and dynamics of biological macromolecules in cells. Furthermore, it discusses the limitations and challenges confronting molecular biosensors and prospects future development directions. "Seeing is believing," and molecular biosensors play a unique role in this regard, enabling unprecedented penetration into the cellular interior to observe biomolecular events and even biological processes, thereby addressing more biological questions.

Full Text

Molecular Biosensors and Molecular Imaging in Cells

Molecular biosensors are sensors constructed from biological macromolecules through genetic recombination or DNA synthesis, capable of real-time, visual detection of key molecular events in living cells and organisms. Currently, the most intensively studied and widely applied molecular biosensors include molecular beacons (MB), resonance energy transfer systems (fluorescence resonance energy transfer and bioluminescence resonance energy transfer), and molecular fluorescence complementation systems (such as bimolecular fluorescence complementation and trimolecular fluorescence complementation). This article in-

troduces the principles and characteristics of these molecular biosensors, with emphasis on their applications in live-cell molecular imaging, such as studying protein-protein interactions in cells and exploring the localization, movement, and dynamics of biological macromolecules in cells. Additionally, we discuss the limitations and challenges facing molecular biosensors and prospect future development directions. "Seeing is believing," and molecular biosensors play a unique role in this regard, enabling us to observe biomolecular events and biological processes inside cells with unprecedented depth, thereby answering more biological questions.

Keywords: molecular biosensor, cell imaging, molecular beacon, fluorescence resonance energy transfer, bioluminescence resonance energy transfer, bimolecular fluorescence complementation

Real-time, visual detection of key molecular events in physiological and pathological processes in living cells and organisms is of great significance for understanding fundamental biological processes and elucidating disease mechanisms. Clinically, disease diagnosis and precision treatment increasingly rely on imaging methods. Molecular biosensors are biomolecular systems that generate visual, quantifiable signals to indicate the occurrence and presence of biological substances or events. They can dynamically trace biomolecular interactions, localization and movement of biological macromolecules, changes in physiological environments, and important biochemical reactions in real time within living cells or organisms. Research over the past two decades has fully demonstrated that molecular biosensors are powerful tools for making molecular events "visible." This review focuses on molecular beacons (MB), resonance energy transfer systems (including fluorescence resonance energy transfer and bioluminescence resonance energy transfer), and molecular fluorescence complementation systems (such as bimolecular fluorescence complementation and trimolecular fluorescence complementation), with emphasis on their principles, characteristics, applications in live cells, and discussion of potential challenges and future directions.

Molecular Beacons

Principle

Molecular beacons were first designed by Tyagi and Kramer [1] in 1996 based on fluorescence quenching principles as stem-loop structured oligonucleotide fluorescent probes, also known as hairpin probes. A classic molecular beacon typically consists of 25–35 nucleotides, including a loop region, a stem region, and attached fluorophore and quencher molecules (Figure 1 [Figure 1: see original paper]). The loop region comprises 15–25 nucleotides that can specifically bind target sequences. The stem region consists of 5–7 complementary base pairs that can reversibly dissociate during molecular beacon binding to target molecules, serving to maintain the specific structure. The fluorophore and quencher are located at the two ends of the stem region [2]. In the absence of target sequences,

the molecular beacon exists in a free state where complementary bases in the stem region bind, forcing the molecular beacon to form a hairpin structure. At this point, the fluorophore and quencher are in closest proximity (approximately 6–10 nm), triggering fluorescence resonance energy transfer: the fluorescence emitted by the fluorophore is absorbed by the quencher and dissipated as heat, resulting in nearly complete fluorescence quenching. Conversely, when target sequences are present, the loop region of the molecular beacon hybridizes with the target molecule, opening the complementary region in the stem. As the fluorophore moves away from the quencher, the molecular beacon' s fluorescence is nearly 100% restored, with fluorescence intensity positively correlated with target molecule concentration in solution.

Applications

Molecular beacons offer high sensitivity, high specificity, and require no removal of redundant probes. While detecting PCR amplification products was the original purpose of molecular beacons, their applications now extend far beyond this. Researchers have successfully constructed various novel molecular beacon probes with superior performance by modifying classic molecular beacon structures, developing diverse fluorophores and quenchers, and coupling nanomaterials (such as gold nanoparticles/graphene, carbon nanotubes, etc.), which have been widely applied in genetic diagnosis, nucleic acid detection, and molecular tracing. This review focuses on molecular beacon applications in live-cell molecular imaging.

The most extensively studied application is the tracing of mRNA in living cells. In 1998, Sokol et al. [3] first demonstrated real-time detection of RNA-DNA hybridization, visualizing 10 mRNA molecules of the proto-oncogene vav under a fluorescence microscope—hybridization signals generated by just 0.1 ag (1 ag = 1$×10^{-18}$ g) of molecular beacons. Subsequently, Bratu et al. [4] pioneered the use of molecular beacons to study mRNA localization and transport in living cells. This team observed the movement of native mRNAs in *Drosophila melanogaster* oocytes and found that genetic manipulation of the 3 untranslated region or chemical perturbation of the microtubule network could significantly alter mRNA distribution. Vargas et al. [5] used molecular beacons to track mRNA molecules in living cells and further visualized the transport behavior of mRNA-protein complexes from transcription sites to nuclear pores, revealing that this transport process depends on enzymatic reactions. Our team pioneered a method for visualizing viral nucleic acids in living host cells using molecular beacon technology, enabling direct observation of the dynamic behavior of poliovirus plus-strand RNA and influenza A virus mRNA under fluorescence microscopy, while combining other experimental approaches to explore viral nucleic acid transport mechanisms [6,7].

Molecular beacons are also commonly used for detecting and tracing molecules such as proteins and ATP in cells, primarily through aptamer beacons formed by molecular beacons and aptamers. Yamamoto et al. [8] first demonstrated

the feasibility of inserting target aptamer binding sites into molecular beacons, constructing a high-affinity aptamer beacon targeting the HIV-1 Tat protein. Compared with traditional molecular beacons, these beacons require only half the number of matching bases (8 bases) with target sequences. Our team employed the same strategy to construct an aptamer molecular beacon specifically recognizing HIV-1 reverse transcriptase, thereby enabling visualization of HIV-1 reverse transcriptase in living cells [9]. Zhang et al. [10] constructed an ATP detection system using molecular beacons, achieving ATP detection in leukemia K562 cells and breast cancer 4T1 cells with an in vitro detection limit as low as (1.1–3.2)$×10^{-7}$ mol/L. Tan et al. [11] and Liu et al. [12] used graphene oxide as a quencher for ATP molecular beacons, obtaining more stable and specific aptamer beacons—the former enabling semi-quantitative detection of intracellular ATP, the latter responding to Ca^{2+}-induced ATP elevation at 5 mmol/L.

Currently, research on developing multifunctional molecular beacons for tracing key molecular events at the single-cell and single-molecule level has emerged. For example, Li et al. [13] designed a dual-functional molecular beacon capable of simultaneously detecting and inhibiting microRNA in zebrafish. Chen et al. [14] used minimally engineered molecular beacons to illuminate single-molecule RNA, employing imaging techniques to deeply investigate the dynamics and localization of long non-coding RNA (lncRNA) in single cells. These directions may become mainstream in molecular beacon applications for cellular imaging.

Resonance Energy Transfer Systems

Resonance energy transfer systems are molecular sensing constructs built using energy transfer principles and genetic manipulation techniques, mainly including fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET).

Fluorescence Resonance Energy Transfer

Principle FRET is a technology for measuring intermolecular distances based on energy transfer, proposed by Förster in 1948 [15]. The basic principle is that when the fluorescence emission spectrum of a donor overlaps with the absorption spectrum of an acceptor and the distance between donor and acceptor is appropriate (generally less than 10 nm), a non-radiative energy transfer occurs. As shown in Figure 2 [Figure 2: see original paper], when the donor is illuminated with excitation light of appropriate frequency, it generates oscillating dipoles that resonate with the dipoles of adjacent acceptors. The energy of the donor fluorophore is non-radiatively transferred to the acceptor fluorophore through dipole-dipole interactions. Overall, FRET requires two simultaneous conditions: (1) the donor molecule must have high quantum yield, with its emission spectrum effectively overlapping the acceptor' s excitation spectrum; (2) both donor

and acceptor molecules must be in an excited state with a distance between 1-10 nm [16].

Characteristics In biological research, FRET technology is a powerful tool for studying biomolecular interactions, particularly protein-protein interactions under physiological conditions in living cells. FRET donors and acceptors are primarily fluorescent proteins, organic fluorophores, and inorganic nanofluorescent materials, requiring external light sources to excite the donor. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are the most commonly used donor-acceptor pair. Since FRET strictly depends on spatial distance (1-10 nm) and most biological molecules fall within this size range, FRET serves as an optical "molecular ruler" for measuring molecular spatial distances, capable of addressing numerous biological questions such as protein dynamics and conformational changes. Unlike classic molecular emission spectroscopy, FRET' s greatest advantage is its use of signal ratios rather than intensity for data analysis, eliminating signal differences caused by sampling errors. In recent years, with the emergence of new fluorescent materials, advances in optical technology, and development of microscopic imaging tools, FRET technology has achieved significant improvements in spatial resolution and detection sensitivity, enabling single-molecule FRET (SmFRET) [17].

Applications FRET has numerous applications in living cells, utilizing spatial information between two protein molecules to answer questions related to molecular movement, protein conformation, and transcriptional regulation. Technically, FRET phenomena are detected through various methods including acceptor photobleaching-induced donor fluorescence enhancement, donor photobleaching, acceptor fluorescence lifetime extension, and donor-acceptor fluorescence intensity ratio methods using microscopy [18]. Here we introduce some representative studies.

(1) Detection of ions under physiological conditions in living cells is a classic application of FRET technology. For example, Myawaki et al. [19] developed a live-cell calcium ion indicator composed of CFP, YFP, calmodulin, and calmodulin-binding peptide M13. The principle is that Ca^{2+} in cells promotes calmodulin binding to M13, thereby increasing fluorescence resonance energy transfer between fluorescent proteins, enabling monitoring and quantification of calcium ions in organelles through FRET detection and data correction. Similarly, Zhang et al. [20] used FRET probes for highly sensitive detection of Hg^{2+} in living cells. Additionally, clever FRET tracking systems have been reported for important physiological parameters such as ATP and pH in living cells [21-23].

(2) FRET is undoubtedly one of the best methods for studying protein-protein interactions. Protein-protein interactions are involved in numerous physiological processes and signal transduction pathways in living organisms, playing key roles in cellular activities. The typical strategy for detecting

protein-protein interactions using FRET technology involves genetically fusing target proteins with a donor or acceptor and expressing them in cells. Under specific spatiotemporal and physiological conditions, if interaction occurs, the donor and acceptor will come into close proximity to generate FRET; otherwise, no FRET occurs. Using this strategy, multiple protein interactions in cells have been identified and traced, such as interactions between high mobility group box 1 protein (HMGB1), Toll-like receptors (TLR), and receptor for advanced glycation end products (RAGE) [24]; interactions between epidermal growth factor receptor (EGFR) and its binding protein Grb2 in subcellular structures [25]; and oligomerization of stromal interaction molecule 1 (STIM1) and its translocation and targeting [26]. Furthermore, FRET is widely used in studies related to RNA detection and protein dynamics in living cells [27-29].

Bioluminescence Resonance Energy Transfer

BRET emerged in 1999 with principles similar to FRET [30]. As shown in Figure 3 [Figure 3: see original paper], the donor in BRET systems is primarily luciferase (Luc). Renilla luciferase (Rluc) uses coelenterazine as substrate, firefly luciferase (Fluc) uses D-luciferin and ATP, and *Gaussia* luciferase (Cluc) uses *Gaussia* luciferin—these are commonly used BRET donors. These substrates and their derivatives are catalyzed and oxidized by corresponding luciferases to emit light. Due to A-B molecular interactions, donor and acceptor come into close proximity. When the distance is 1–10 nm, BRET occurs and the acceptor emits fluorescence. Under substrate saturation, acceptor fluorescence intensity is positively correlated with enzyme concentration.

Compared with FRET, since BRET does not require external light excitation of the donor, it can avoid photobleaching, light absorption, and tissue damage caused by strong light sources, while reducing background signals from biological autofluorescence [31]. In live-cell imaging applications, BRET is largely similar to FRET, such as in protein-protein interaction studies, ATP detection, and protein activity assays [32-35]. Notably, in tissues and living organisms rich in blood cells or hemoglobin, BRET has significant advantages in signal-to-noise ratio due to reduced light absorption and autofluorescence in biological systems [36].

Molecular Fluorescence Complementation Systems

Molecular fluorescence complementation systems are molecular sensing constructs built using protein fragment complementation technology. This review focuses on fluorescence protein-based molecular complementation systems, including bimolecular fluorescence complementation (BiFC) and trimolecular fluorescence complementation (TriFC).

Bimolecular Fluorescence Complementation

Principle BiFC originated from protein fragment complementation technology, first reported by Ghosh et al. [37] in 2000. As shown in Figure 4a [Figure 4: see original paper] [38], fluorescent proteins are split at specific regions (typically loop regions) through genetic splicing to form two non-fluorescent fragments called N-fragment and C-fragment. The N- and C-fragments are fused via linker peptides to a pair of interacting target proteins. Due to interactions between the target protein pair, the N- and C-fragments of the fluorescent protein come into close proximity, achieving spatial complementation, followed by molecular reconstruction to restore conformation and produce fluorescence under appropriate excitation wavelengths. Conversely, if no interaction exists between target proteins, no fluorescence is produced.

Characteristics BiFC can be applied both in vivo and in vitro, featuring high sensitivity and low noise. BiFC molecular elements are pre-introduced into cells through genetic manipulation for expression, allowing direct fluorescence observation of biomolecular interactions and their spatial location information in living cells or organisms without exogenous addition, thus avoiding interference and observation artifacts caused by external substances. Compared with FRET and BRET technologies, BiFC does not require complex data processing and can be performed with conventional fluorescence microscopes. Using different colored fluorescence complementation systems enables simultaneous detection of multiple protein interactions in living cells. Given these characteristics, BiFC is widely used in studying biomolecular interactions, particularly interactions within cells and between cells and environmental substances.

Currently reported BiFC systems are typically irreversible—once reconstituted, they are difficult to reopen. This feature gives BiFC high sensitivity, capable of detecting weak interactions with KD approaching 1 mmol/L, but also hinders monitoring dynamic changes in protein-protein interactions. To address this, Tchekanda et al. [39] developed a reversible BiFC system using a genetically modified near-infrared fluorescent protein mutant monomer IFP1.4, further expanding BiFC applications.

Applications BiFC is commonly used to study protein oligomerization. Among these, misfolding-oligomerization and fibrillation of α-synuclein are considered central events in the development and progression of Parkinson' s disease and related neurodegenerative disorders. Outeiro et al. [40] directly observed α-synuclein oligomerization in living cells using BiFC and found that protein oligomerization-induced cytotoxicity could be alleviated by Hsp70. Additionally, G protein-coupled receptor (GPCR) oligomerization is another important research area. GPCR-related signal transduction is involved in numerous diseases, and GPCR oligomerization plays important roles in regulating receptor pharmacology and function—currently, approximately 30%–40% of drug cellular targets are designed against GPCRs [41,42]. In this field, numer-

ous studies and discoveries based on BiFC have been reported, including GPCR dimerization [43] and inhibition of dimerization [44], distribution and plasma membrane assembly of adenosine A2A receptors [45,46], and pharmacological studies of neuropeptide Y Y1/Y5 receptor heterodimers using GPCR dimer imaging [47]. Ciruela and Vilardaga [48] comprehensively summarized BiFC applications in GPCR research. Therefore, BiFC is not only an important tool for tracing protein oligomerization but also an effective means for identifying disease therapeutic targets and drugs.

BiFC is also widely used to study virus-host interactions, which are significant for understanding viral life cycles and pathogenic mechanisms. For example, Hemerka et al. [49] used BiFC to reveal previously unknown interactions between influenza virus polymerase complex subunits PA and PB2, and conducted detailed analysis of PA-PB2 binary complex subcellular localization and nuclear import—this research framework provides a basis for further exploring the biological relevance of PA-PB2 interactions with influenza virus polymerase activity and viral replication. Currently, human virus studies using BiFC also involve characterization of Epstein-Barr virus latent membrane protein 1 [50], autophagy inhibitor drug screening based on Beclin1-Bcl2 complex dissociation [51], and interactions between HIV-1 non-structural protein Vpr molecules [52].

Development of Molecular Complementation Systems

Far-Red and Near-Infrared BiFC In mammals, there exists a near-infrared (NIR) optical window (650–900 nm) that avoids light absorption by water, hemoglobin, and other molecules in organisms [53,54]. To address this issue, our team developed a series of long-wavelength fluorescence complementation systems using fluorescent proteins such as mCherry (λex/λem = 587 nm/610 nm) and mNeptune (λex/λem = 600 nm/650 nm) [55,56]. Furthermore, to better achieve deep tissue imaging, we broke the convention of using GFP-like fluorescent proteins to build BiFC and innovatively developed a near-infrared BiFC system based on bacterial phytochrome iRFP (λex/λem = 690 nm/713 nm). This work provides a powerful tool for studying protein interactions under physiological conditions and offers new strategies for drug evaluation in living cells [57].

Trimolecular Complementation Systems Given unknown protein-RNA interactions in cells, TriFC systems based on BiFC have emerged. As shown in Figure 4b, in the TriFC system, the C-fragment of the fluorescent protein attaches to reporter mRNA through the interaction between a known protein (A) and RNA (D). The N-fragment is fused with RNA-binding protein B . If the RNA-binding protein interacts with the target sequence in the reporter mRNA, the N- and C-fragments come into close proximity to restore fluorescent protein conformation and produce fluorescence [58]. TriFC can be used not only to identify RNA-protein interactions but also to analyze the localization and dynamics of RNA-protein interactions in living cells. Numerous studies

have validated the utility of this method [59-61]. Our team used TriFC to focus on studying interactions between influenza virus and HIV-1 mRNAs and host proteins, elucidating related biological mechanisms [55,62].

Recently, our team also developed a three-fragment fluorescence complementation system (TFFC) for imaging protein trimers. We split the fluorescent protein mIrisFP with photo-conversion and photo-activation properties into three fragments, combined with super-resolution imaging to trace interactions between G protein trimer subunits at the single-molecule level in living cells [63]. Additionally, molecular complementation systems formed by "splitting between the 10th and 11th β-strands of fluorescent proteins" (split fluorescent proteins complementation) have been used for live-cell visualization studies such as protein localization and cytoplasmic peptide delivery [64-66].

Conclusions and Prospects

Over the past two decades, the aforementioned fluorescence-based molecular biosensor technologies have advanced rapidly, with numerous related research papers published covering materials, chemistry, physics, biomedical science, and life sciences. From the perspective of live-cell applications, using multicolor labeling combined with super-resolution imaging for real-time dynamic observation of multiple key molecular events at the single-cell and single-molecule level will become mainstream. However, deep applications of these molecular biosensors in tissues, organs, and living organisms still face severe challenges. Improving signal-to-noise ratio, avoiding photon absorption by biological substances, and enabling long-term tracking remain difficult problems. There is an urgent need to create fluorescent probes with good biocompatibility, high brightness, and photostability, develop ultra-high sensitivity sensing systems, improve imaging data algorithms, and further advance optical technologies and imaging tools. With technological progress, molecular biosensors will enable us to obtain deep-level information about life processes in real time with unprecedented capability, thereby answering a series of important fundamental questions in life sciences and expanding into clinical applications to serve human health.

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Wang Dianbing, Ph.D., Associate Professor at the Institute of Biophysics, Chinese Academy of Sciences. Her long-term research focuses on biosensors and analytical pathogen microbiology. She has published over 20 SCI-indexed papers and holds 7 Chinese patents. She has led and participated in multiple projects funded by the National Natural Science Foundation of China, the "863" Program, and the Chinese Academy of Sciences, and was awarded the First Prize of Natural Science in Hubei Province. E-mail: wangdb@moon.ibp.ac.cn

Cui Zongqiang, Ph.D., Researcher at the Wuhan Institute of Virology, Chi-

nese Academy of Sciences. He became a full professor at the Wuhan Institute of Virology in 1993, specializing in biosensors, nanobiology, and analytical microbiology. He has published approximately 240 peer-reviewed papers (200 SCI-indexed) and authored 3 monographs on biosensors and biochips. He previously engaged in macro-management of basic research at the Department of Basic Research, Ministry of Science and Technology. He currently serves as Vice President of the Chinese Society of Biotechnology, Co-chair of the Division of Nanobiotechnology, Biosensors and Biochips of the Asian Federation of Biotechnology (AFOB), and Chinese Representative to the APEC Meeting of Chief Science Advisors (2013, 2015, and 2016). In 2015, he was awarded an Honorary Doctor of Science degree by the University of Alberta, Canada. E-mail: zhangxe@ibp.ac.cn

Note: Figure translations are in progress. See original paper for figures.