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Optical Sensors to Gain Mechanistic Insights into Signaling Assemblies

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Abstract

Protein complexes play a major role in transducing information from outside the cell into instructions for growth and survival, and understanding how these complexes relay and shape intracellular signals has been a central question in signaling biology. Fluorescent proteins have proven paramount in opening windows for researchers to peer into the architecture and inner workings of signaling assemblies within the living cell and in real-time. In this review, we will provide readers with a current perspective on the development and use of genetically encoded optical probes to dissect the function of signaling complexes.

Introduction

Signaling networks are essential for cells to sense their environment and rapidly translate external information into decisions that promote growth and sustainability. Due to the complexity of the intracellular space, these networks have evolved multimolecular assemblies to facilitate signal transduction by specifically positioning enzymes and substrates for interaction [1]. Extensive biochemical characterization over the last few decades has provided researchers a glimpse into the assembly of signaling complexes, or signalosomes, and the mechanisms through which they shape signals. However, obtaining a more complete understanding of intracellular communication requires tools that are capable of capturing the spatiotemporal dynamics of these processes within individual living cells.

Recent technological advances in live-cell imaging and the landmark discovery of fluorescent proteins (FPs) have revolutionized the signaling field and led to the development of genetically encoded biosensors for interrogating signaling in the native biological context

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as opposed to in the test tube [2]. In addition, these tools have opened up the possibility of monitoring individual cells at the time scale at which signaling events occur, whereas bulk measurement techniques often fall short of accurately describing the cell-to-cell heterogeneity of signaling, i.e., an average response can mask important dynamics, such as oscillations and transient spiking [3]. Further adaptations of these tools over the last ten years have promoted the design of fluorescence-based biosensors that can actually provide mechanistic descriptions of signaling assemblies by measuring localized protein-protein interactions (PPIs), second messenger concentrations, and enzymatic activities [4].

In this review, we will briefly cover some of the optical-based tools that are currently utilized to probe multiprotein signaling complexes. The technologies covered here are not exhaustive, but will rather focus on a few important techniques and the present state of probe and sensor development.

Assembly Architecture

Signaling complexes can be assembled from many different enzymes, adaptors, and scaffolds, forming a localized, information-rich protein interaction network. The components within the assembly can exist in many physiologically relevant molecular states, such as being bound to the complex, being chemically modified, or adopting a specific conformation [1]. Perturbations to the composition of an assembly, for example, by mutations, often transform signaling pathways and can cause detrimental effects such as cancer and Alzheimer's disease [5,6]. To understand the signal-shaping mechanisms of these multiprotein machines, it is important to characterize the dynamic PPIs in the local context of the living cell. Below, we outline a few technologies that have assisted researchers in dissecting the architecture of signaling complexes and highlight current developments that push the limits of measurement.

From revealing the composition of a signaling complex to measuring the dynamic changes within a signaling complex, Förster Resonance Energy Transfer (FRET) is a powerful tool for probing PPIs. FRET involves the non-radiative transfer of energy from an excited donor fluorophore to an acceptor fluorophore. This photophysical process is immediate and functions effectively in the 1–10 nm regime with an inverse (distance)⁶ dependence, making FRET a sensitive method to study interactions at the scale of macromolecules in situ (Figure 1a) [7]. Advancements in imaging/detection techniques and the discovery of spectral FP variants have led to the routine utilization of FRET in signaling research.

Many signaling interactions occur quickly and reversibly, and thus require dynamic, reversible probes. Non-covalent interactions can be effectively coupled to the distance-dependent FRET signal by directly fusing or tagging the proteins of interest to fluorescent proteins [8]. This methodology has been utilized to build signaling network models. For example, the G-protein signaling pathway comprises many PPI events, including receptor activation, G-protein dissociation, and receptor recycling; almost every ligand-induced event in this pathway has been monitored using FRET [7,8]. In addition, many of the players undergo conformational changes upon binding or unbinding, and FRET can be used to measure these changes in an intramolecular fashion. For example, ligand-inducible changes

of GPCRs can be monitored by inserting CFP into an intracellular loop and placing YFP at the C-terminus [11]. The G-protein pathway has also benefitted from BRET, another resonance energy transfer-based method that utilizes a bioluminescent luciferase as the energy donor, obviating the need for potentially damaging exogenous illumination and leading to less photobleaching and lower background [12].

The finite range of the visible spectrum and the broad excitation and emission spectra of FPs can make FRET a spectrally expensive technique [13]. Fortunately, alternative single-color methods can also be employed. The EGF receptor is known to dimerize and oligomerize upon stimulation, but only recently has a method for monitoring real-time changes in multimerization been reported [14]. This technique, known as homo-FRET, involves FRET between identical fluorophores and can be quantified by measuring the polarization of emitted light at a single wavelength relative to the excitation light [13,14]. Homo-FRET has been successfully applied to measure receptor clustering by labeling the EGF receptor with a single, monomeric GFP and measuring the loss of fluorescence anisotropy [17]. Live-cell imaging using this single-color technique led to the discovery that EGFR dimerization is the result of receptor activation instead of the converse [14].

Signaling complexes and signalosomes typically contain several physiologically important PPIs that are also incredibly dynamic. To monitor multiple interactions within a complex simultaneously, researchers are extending hetero-FRET for use with multiple donors and acceptors. For example, T-cell activation and subsequent cytoskeletal rearrangement is initiated by a multiprotein assembly comprised of the GEF Vav1 and adaptor proteins Nck and SLP-76 [18]. Immunoblotting revealed the identities of the key components, but the specific PPIs and the dynamics governing complex formation remained elusive. Utilizing the distance dependence of FRET to monitor extremely local interactions in real-time, Pauker et al. fused three spectrally distinct FPs to the three components [18]. In order to detect all interactions after T-cell activation, an imaging scheme was needed to subtract spectral bleed-through and account for indirect FRET through an intermediate acceptor/donor. This study demonstrated that Nck and Vav1 exist as a constitutive heterodimer and that activation triggers the binding of SLP-76 to Nck. This powerful multi-color FRET imaging approach to monitor many interactions simultaneously has been proposed to work with *N* spectrally distinct FPs, provided the appropriate corrections and controls are applied [17,18].

Despite its sensitivity to small changes in distances, FRET can be limited by poor signal-to-noise ratios and the requirement that a large subset of FPs must associate to produce a detectable signal [19,20]. These restrictions tend to hamper the study of very weak/transient and/or rare interactions [21,22]. For example, the binding of effectors to tyrosine kinases via SH3 domains regulates many important cell processes; however, these interactions tend to be short-lived, making FRET a less-than-optimal detection method [23,24]. An alternative technique involves the creation of a fluorescent signal when two proteins are in close proximity. By splitting a fluorescent protein into two non-fluorescent fragments and fusing each to an SH3 domain and a binding effector, heterodimerization can be measured and imaged by the reconstitution of the FP and the gain of fluorescence (Figure 1b) [25,26,27]. Although the irreversibility of this process and the time required for fluorophore maturation prevents detailed dynamic measurements, bimolecular fluorescence complementation

(BiFC) is well-suited for visualizing weak interactions within the cell [27]. Multiple BiFC-capable FP variants are also available and have been successfully utilized to image many PPIs, including homo- and heterodimers of the adenosine and dopamine receptors simultaneously [30].

Increasing evidence suggests that signaling assemblies are spatially organized at the nanometer scale; however, the spatial resolution of the above methods is limited by the diffraction of light (~250nm) [29,30,31]. In order to map PPIs at a resolution commensurate to their molecular scale, BiFC has been extended to incorporate photo-activatable FPs for super-resolution imaging. For example, the GTPase Ras has been shown to exist in functionally-important “nanoclusters,” but the organization of Ras-binding effectors, such as Raf, is poorly understood [34]. Recently, a BiFC-compatible split version of photo-activatable mCherry was generated, and each end was fused to either Ras or Raf [35]. Both standard Photo-activated Localization Microscopy (PALM) imaging and single-particle tracking were used to map the clustered heterodimers and their collective motion. However, the inherent tradeoff between spatial and temporal resolution prohibited the extraction of meaningful dynamic information using BiFC-PALM. A recently reported super-resolution BiFC technique sought to strike a balance and permit live-cell detection of PPIs. Utilizing a split photoblinking Venus, the functionally-relevant association between the calcium store sensor Stim1 and the store-operated calcium channel Orai1 was mapped using reconstituted fluorescence-based stochastic optical fluctuation imaging (refSOFI) [36].

In certain applications, the only pertinent information for researchers is the inclusion or absence of specific effectors within an assembly. The high signal-to-noise ratio of BiFC thus permits cellular screening to probe assembly architecture [37]. For example, the important regulatory kinase PKB/Akt functions in cell growth, proliferation, and apoptosis; however, not all of the bound effectors of PKB have been characterized [38]. In a first-of-its-kind experiment, a split-GFP screen to identify novel effectors of PKB was performed by fusing one GFP fragment to a cDNA library of “prey” proteins, and fusing the other fragment to PKB. By screening thousands of “prey” proteins, researchers found one, Ft1, that binds to PKB and promotes the association of PKB with PDK1 [39]. Due to the involvement of PPIs in many diseases, there has also been a large push to develop high-throughput assays for drug development. A recently described high-content screen to find inhibitors of the activity-dependent dimerization of an HIV-1 accessory factor using split YFP identified several hit compounds [40]. Improvements in BiFC for screening continue and include a tripartite fluorescence complementation assay to both study ternary complexes and reduce background complementation, as well as a split near-infrared FP to allow deep-tissue detection [39,40,41].

Assembly-Based Signaling Functions

Many signaling networks rely on signalosome formation in order to “reshape,” or modify, downstream dynamics and optimize specific enzymatic reactions [1]. In the previous section, we looked at methods to dissect the components and interactions within an assembly. In this section, we will focus on methods, primarily using biosensors, to study the mechanisms by which signalosomes regulate signaling pathways.

Genetically Encoded Biosensors

Genetically encoded biosensors have become a valuable tool in allowing researchers to measure the spatiotemporal dynamics of intracellular signals within living cells. Their strength lies in their ability to specifically monitor changes in biochemical activities in real-time and with minimal invasiveness to the cell [42,43]. Biosensors generally comprise two parts: a sensing component and a reporting component [44,45]. The sensing component is responsible for detecting the relevant biochemical change through either a binding/unbinding event or protein modification, leading to a conformational change. The reporting component translates the conformational change into a detectable signal, most often via fluorescence or bioluminescence. For example, the genetically encoded calcium sensor Cameleon utilizes a calcium-dependent conformational switch composed of calmodulin and a fragment of myosin light-chain kinase sandwiched between a CFP/YFP FRET pair (Figure 2a) [48]. This same sensing unit has also been used to generate a collection of high-contrast, single-color probes that have gained in popularity (Figure 2b) [47,48]. In addition, an entire suite of kinase activity biosensors have been engineered by fusing a kinase-specific substrate domain to a phosphoaminoacid binding domain and flanking this switch between a FRET pair (Figure 2c) [46]. Phosphorylation of the substrate domain triggers a conformational change that induces a FRET change.

Biosensors to Study Signalosome Functions

To directly probe localized signaling within a signalosome, biosensors are frequently fused to a component of the multiprotein complex. Many signaling networks include scaffold and/or adaptor proteins that organize PPIs within an assembly, and biosensors have helped uncover a set of design principles complexes utilize to modify signal behavior. For example, fusing a FRET-based reporter for PKA activity (AKAR) to a class of PKA anchoring proteins (AKAPs) revealed an increase in activity acceleration and amplitude upon PKA stimulation, demonstrating that scaffolds can create kinetically distinct microdomains (Figure 3) [49,50]. Furthermore, a recent AKAP-fused biosensor study revealed that the activity of AKAP-bound protein kinase C (PKC) is not only accelerated and amplified but is also “insulated” from competitive inhibitors, as compared with general PKC activity at the plasma membrane. AKAPs and other scaffold proteins have also been shown to interact with many additional signaling proteins, such as adenylyl cyclases, phosphodiesterases (PDEs), and phosphatases, and can increase the specificity of certain signals [53]. For example, deletion of the PDE-binding domain in a particular AKAP was shown to reshape the stimulated PKA activity from a transient response into a more sustained response [51]. Biosensors are indispensable tools in dissecting the mechanisms complexes use to modify intracellular signaling and precisely control the spatiotemporal dynamics of signaling cascades.

Many groups are continuously pushing the boundaries of genetically encoded biosensor design. Due to their reliance on fluorescence or bioluminescence, the challenges facing biosensors are similar to the limitations of tools for probing assembly architecture, namely, limits of spectral space and signal intensity. As mentioned previously, a set of new near-IR

FPs have been optimized and shown to be suitable as FRET acceptors for in vivo reporting [52,53].

Despite finite spectral space, other groups have pushed the limits of multiplexing by co-expressing many biosensors and using innovative multi-dimensional unmixing methods [56]. The use of single-color biosensors frees much of the visible spectrum but often at the cost of losing a ratiometric measurement. However, a new class of biosensors utilizing homo-FRET permits a single-color, ratiometric readout by measuring changes in the steady-state fluorescence anisotropy [57]. Bottlenecks for biosensor development typically reside in the initial design and optimization stages of a new reporter; however, many groups have turned to higher-throughput screening procedures to rapidly test thousands of sensor/FP variants and evolve these mutants into better sensors. Lysate and colony screens, as well as microfluidic-based approaches, are becoming a mainstay in the development pipeline, resulting in probes with increased dynamic range and enhanced sensitivity [56,57].

Conclusions and Future Perspectives

Although far from exhaustive, this review highlights a few optical methods for probing signaling assemblies. From monitoring PPIs to interrogating the functional roles of multiprotein complexes, genetically encoded tools allow researchers to measure the spatiotemporal signatures of specific signaling networks in living cells and tissues.

Despite the high sensitivity and specificity of biosensors, the ability to report biological changes is sometimes hampered by overexpression, and thus requires a number of control experiments. Stably expressing cell lines and/or endogenously-fused sensors could alleviate such concerns. Recent progress in employing CRISPR/Cas9 for gene knockin has spurred interest in the development of endogenously expressed sensors for PPIs and activity [60].

The boundaries for attaining higher-resolution maps of interactions and activities within the cell are constantly being pushed. As mentioned previously, new methods such as BiFC-PALM and refSOFI allow researchers to image PPIs below the diffraction limit. In addition, the heterogeneity in the composition and mechanistic function of multiprotein assemblies suggests the need to study complexes at the single-molecule level instead of adopting ensemble approaches. Newer techniques such as single-molecule pull-down (SiMPull) provide a platform for extracting individual protein complexes from cells and tissues for single-molecule investigation [61]. The advent of brighter organic fluorophores and protein-tagging systems such as SNAP and Halo could also offer the possibility to utilize single-molecule FRET to directly probe individual signaling complexes [62]. In addition to improvements in spatial resolution and single-molecule detection, progress in attaining higher temporal resolution for biosensors have afforded researchers the ability to measure extremely transient signals such as calcium spikes and action potentials [61,62].

Fluorescence and bioluminescence-based tools for studying macromolecular complexes and signalosomes have contributed greatly to our understanding of intracellular signaling. Persistent innovation, including better fluorophores and robust tool design, will continue to

allow researchers to design comprehensive and detailed experiments to probe the spatiotemporal dynamics of assembly-based signaling.

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Highlights

- Signaling complexes have distinct assembly architectures and fundamentally change signaling dynamics within the cell.
- The interactions between a complex's components can be monitored in real-time using optical methods such as FRET and BiFC.
- Genetically encoded biosensors measure changes in biological activity and can be used to study signaling *in situ*.

Figure 1.

A signaling complex's internal architecture can be probed by tagging components with fluorescent proteins (FPs). As schematically shown in **(a)**, two interacting proteins, such as a GPCR and β -arrestin, are tagged with FPs capable of FRET. BiFC can also be utilized to detect PPIs, as shown in **(b)**. Two nonfluorescent FP fragments are fused to an interacting protein pair, such as an SH3 domain and an effector, and complex formation triggers the reconstitution of the fluorescent signal.

Figure 2.

FP-based, genetically encoded biosensors are used to measure changes in signaling activities and concentrations. In (a), the FRET-based calcium reporter Cameleon works by binding calcium via a calmodulin domain (CaM) and triggering binding to a domain derived from myosin light chain kinase (M13). This conformational change then elicits FRET by bringing two fused FPs into proximity. As shown in (b), a suite of single-color calcium reporters, known as GCaMPs, have been developed that utilize the same CaM and M13 domains but fused to different parts of a circularly-permuted GFP. Calcium binding modulates the GFP's

fluorophore, leading to increased fluorescent emission. Kinase activity can also be sensed using similar design principles as Cameleon, as seen in (c). The sensing unit is comprised of a kinase-specific substrate domain that, once phosphorylated, binds to a general phosphoaminoacid binding domain (PAABD), and triggers a change in FRET.

Figure 3.

Signaling complexes tend to fundamentally alter dynamics within a signalosome. Many important signaling enzymes, such as the cAMP dependent kinase PKA, are localized throughout the cell by interacting with scaffold proteins as well as with a variety of additional effectors. Genetically encoded biosensors can measure signaling dynamics both within these signalosomes and in the cytosol. In the case of PKA, it has been shown that stimulated PKA activity is both accelerated and amplified within a PKA anchoring protein (AKAP)-centric signalosome, as compared to outside this complex.