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Review

Förster Resonance Energy Transfer — An approach to visualize the spatiotemporal regulation of macromolecular complex formation and compartmentalized cell signaling

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ABSTRACT

Background: Signaling messengers and effector proteins provide an orchestrated molecular machinery to relay extracellular signals to the inside of cells and thereby facilitate distinct cellular behaviors. Formations of intracellular macromolecular complexes and segregation of signaling cascades dynamically regulate the flow of a biological process.
Scope of review: In this review, we provide an overview of the development and application of FRET technology in monitoring cyclic nucleotide-dependent signalings and protein complexes associated with these signalings in real time and space with brief mention of other important signaling messengers and effector proteins involved in compartmentalized signaling.
Major conclusions: The preciseness, rapidity and specificity of cellular responses indicate restricted alterations of signaling messengers, particularly in subcellular compartments rather than globally. Not only the physical confinement and selective depletion, but also the intra- and inter-molecular interactions of signaling effectors modulate the direction of signal transduction in a compartmentalized fashion. To understand the finer details of various intracellular signaling cascades and crosstalk between proteins and other effectors, it is important to visualize these processes in live cells. Förster Resonance Energy Transfer (FRET) has been established as a useful tool to do this, even with its inherent limitations.
General significance: FRET technology remains as an effective tool for unraveling the complex organization and distribution of various endogenous signaling proteins, as well as the spatiotemporal dynamics of second messengers inside a single cell to distinguish the heterogeneity of cell signaling under normal physiological conditions and during pathological events.

1. Introduction

Cell signaling is a multifaceted process involving numerous signaling messengers and effector proteins [1]. To understand overall cellular behavior, it is important to concentrate on the pattern, duration and preciseness of a signaling cascade in response to cues [2]. Small, yet elegant molecules such as cAMP and cGMP play critical roles as second messengers in carrying out a precise signaling cascade with the right specificity [3–5]. The spatiotemporal regulation of the intracellular dynamics of these cyclic nucleotides can determine the duration and localization of cellular responses and segregates the global network of cell-signaling systems into separate cellular compartments. To understand the localized modulation of cyclic nucleotides, there is a need to focus on the mechanisms by which a cell maintains different cyclic nucleotide environments at different subcellular regions. Together with synthesis and catabolism by different isoforms of phosphodiesterases, nucleotide transporters such as multidrug resistance protein 4 and 5 (MRP4/5) are responsible for determining intracellular concentrations of cAMP and cGMP and the subsequent regulation of different cyclic nucleotide-dependent signalings [2, 6–13]. Cyclic nucleotide-dependent and -independent kinases, such as protein kinases A, G and C, facilitate compartmentalized signaling by restricted activation of specific substrate populations in distinct cellular compartments [14–18]. Other important signaling proteins, including the Ras superfamily of small guanosine triphosphatases (GTPases), also are dynamically regulated in time and space in order to generate discrete and localized cellular effects. The cycles of activation by guanine nucleotide exchange factor (GEF) and inactivation by GTPase-activating protein (GAP) finely control segregated interactions of

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GTPase with downstream effectors [19–21]. Precise measurement of free cytosolic and organellar Ca^{2+} is important to understand the highly localized Ca^{2+} signaling required for allosteric regulation of a myriad of signaling proteins, and cells provide a collection of components for managing the wide range of Ca^{2+}-dependent spatial and temporal signals [22]. Nevertheless, the interactions of receptors either between themselves or with their downstream targets are both indispensable for initiating a signaling cascade and an early hallmark step for a cell response to external cues [23,24].

The regulation of signaling machineries occurs in a highly compartmentalized fashion, and a fine-tuned localized modulation near the target effector rather than a global signaling wave directs the specific signaling flow [2,25]. For example, although a macromolecular complex containing CFTR and MRP4 at the apical membrane regulates the secretory properties of gut epithelia, this interaction plays an important role for β-adrenergic-stimulated contraction in cardiac myocytes, and both of these occur via the compartmentalized alteration of cAMP dynamics [26,27]. In the past few decades, efforts have been made to better understand compartmentalized cell signaling and FRET technology, which is a quantum mechanical phenomenon that relies on spectral overlap between donor and acceptor molecules, provides a method to do so [28–30]. After being first theorized by Dr. Theodor Förster in 1948, it was more than 50 years before FRET was used in the field of cell biology for direct visualization and quantification of biological function [29,31–34]. The spatial and temporal resolution of a signaling flow via modulation of secondary messengers or through the formation of macromolecular complexes provides more-vivid information about complex cell-signaling phenomena in real time. The focus of this review is a comprehensive exploration of FRET technology used to understand compartmentalized cell signaling.

2. Ratiometric and Direct/Sensitized FRET

FRET is the transfer of excitation energy of an electronically excited donor fluorophore to a nearby acceptor chromophore through a long-range dipole–dipole interaction and without the emission of photons [35,36]. The spectral overlap and the dipole orientations of the donor and acceptor molecules, together with the distance between chromophores, determine the efficiency of FRET and it is inversely proportional to the sixth power of the distance between chromophores [37]. The distance dependency (<10 nm) of FRET signal makes it a useful tool for measuring proximity and conformational changes of biological molecules as a spectroscopic ruler with a higher spatial resolution compared to conventional optical microscopy [37–39]. Typically, fluorescence signals are measured for cells expressing donor or acceptor or both molecules, and FRET efficiencies are calculated [40]. Two types of FRET signals can be monitored for this purpose; i) Ratiometric FRET, where the ratio between the intensites of donor and acceptor or FRET and donor was measured, and ii) Direct/Sensitized FRET, where the acceptor emission was measured upon the excitation of the donor. Ratiometric FRET is the most-convenient readout when the donor and acceptor are stoichiometrically fixed and fused in a single polypeptide chain. For Ratiometric FRET, the Ratio Numerator and Ratio Denominator determine the channel specificity [26,41,42], whereas for Direct/Sensitized FRET, the FRET channel must use a donor exciter and acceptor emitter [43–45]. An alternate intensity-based assay measuring donor dequenching after acceptor bleaching provides specific and accurate static information about FRET efficiency, but this assay is limited to single measurement due to its destructive nature. The nondestructive and fluorescence-decay kinetics-based methods, such as detection of change in the excited-state lifetime of the donor molecule, still need further development [29,38]. For example using the fluorescence lifetime imaging microscopy (FLIM) technique, the decay kinetics of a chromophore is measured in a nanosecond timeframe. Though this technique can spatially resolve various physiological parameters in live cells independent of the chromophore concentration, temporal resolution still remains as a major limitation associated with FLIM [46,47].

3. FRET to study compartmentalized cyclic-nucleotide signaling

In recent years, Ratiometric FRET technology has been successfully exploited to monitor the spatially restricted dynamics of a variety of cellular messengers, such as cAMP, cGMP and Ca^{++}, and to visualize the flow of a signaling cascade in real time [48–51]. In this review, we emphasize the development and application of FRET-based sensors to resolve the spatiotemporal dynamics of cyclic nucleotides and cyclic nucleotide-dependent signaling in healthy and diseased cells. In cells, cAMP and cGMP are synthesized by adenylyl cyclase (AC) and guanylate cyclase (GC), respectively, and both cytosolic and membrane-bound forms of these two enzymes have been identified [18,52,53]. After synthesis, these cyclic nucleotides either degrade to their corresponding monophosphates by the action of different isoforms of phosphodiesterases (PDEs) or efflux out of the cell by the action of endogenous membrane transporters, such as MRP4/5 [2,11,13]. Among the eleven isoforms of PDEs, PDE 4, 7 and 8 are cAMP specific and PDE 5, 6 and 9 are cGMP specific, whereas other isoforms exhibit dual specificity [13,54]. The distinct localizations and specificities of the enzymes maintain the discrete microdomains of cyclic nucleotides in response to different intrinsic and extrinsic signals. The spatial organization of different effector molecules by the scaffolding proteins also assists the establishment of the specific compartments where MRP4/5, together with several isoforms of PDEs, play pivotal roles in maintaining the localized restricted diffusion of these second messengers, instead of a rapid and global modulation, to induce a more-specific response [2].

In biology, the most-commonly used fluorophore, green fluorescence protein (GFP), has inherent limitations, such as slow rotation and spatially-restricted behavior, that make it unsuitable for use as a FRET indicator in bioimaging [55,56]. However, spectral mutants of GFP, cyan (CFP) and yellow (YFP), form the best pair of FRET fluorophores [29,57]. To visualize the spatiotemporal dynamics of cAMP, fluorophores have been attached to the cAMP-dependent protein kinase A (PKA) subunit. Unlike the bimolecular Ca^{++} indicator 'Cameleon' that consists of CFP-calmodulin and calmodulin-binding peptide M13-YFP and gives a larger FRET signal upon Ca^{++} binding [49,58,59], binding of cAMP to the PKA-regulatory subunit induces the release of catalytic subunits and therefore disrupts the FRET signal, followed by an increase in the donor-to-FRET ratio [51,60]. Using the PKA-based FRET indicator, it has been determined that migrating cells have polarized accumulation of cAMP at the leading edge [61], and recently we have shown that inhibition of MRP4 augments the polarized cAMP concentration at the leading edge of a migrating fibroblast and thus facilitates cell migration [41]. To overcome the variability in the expression of different subunits and endogenous unlabeled-molecule-mediated error in FRET detection, the need for a unimolecular fluorescent indicator arises. The cAMP-dependent conformational change in exchange protein directly activated by cAMP (EPAC) makes it a readily targetable FRET-based sensor for cAMP by fusing the N terminus of EPAC with CFP and the C terminus with the YFP analogue [62]. Upon cAMP binding, EPAC undergoes a conformational change to liberate the catalytic domain, which moves CFP and YFP apart from each other to cause a reduced FRET signal [26,27,41]. Considering the relatively larger conformational change induced by cGMP and an almost 100-fold higher selectivity for cGMP compared to cAMP, cGMP-dependent protein kinase (PKG) has been modified and flanked by CFP and an improved pH-insensitive YFP variant. Thus, an efficient genetically encoded FRET-based cGMP sensor called Cygnet 2.1 has been developed to visualize cGMP regulation in real time. Cygnet 2.1 undergoes a 1.4- to 1.5-fold increase in cyan-to-yellow emission upon saturation, and this dynamic range is comparable to the cAMP sensor [50,63]. The mechanism of FRET sensors specific for cAMP and cGMP is illustrated in Fig. 1. These