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Review article

Genetic biosensors for imaging nitric oxide in single cells☆

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SHORT ABSTRACT

Over the last decades a broad collection of sophisticated fluorescent protein-based probes was engineered with the aim to specifically monitor nitric oxide (NO), one of the most important signaling molecules in biology. Here we report and discuss the characteristics and fields of applications of currently available genetically encoded fluorescent sensors for the detection of NO and its metabolites in different cell types.

Long abstract: Because of its radical nature and short half-life, real-time imaging of NO on the level of single cells is challenging. Herein we review state-of-the-art genetically encoded fluorescent sensors for NO and its by-products such as peroxynitrite, nitrite and nitrate. Such probes enable the real-time visualization of NO signals directly or indirectly on the level of single cells and cellular organelles and, hence, extend our understanding of the spatiotemporal dynamics of NO formation, diffusion and degradation. Here, we discuss the significance of NO detection in individual cells and on subcellular level with genetic biosensors. Currently available genetically encoded fluorescent probes for NO and nitrogen species are critically discussed in order to provide insights in the functionality and applicability of these promising tools. As an outlook we provide ideas for novel approaches for the design and application of improved NO probes and fluorescence imaging protocols.

1. Introduction

Extensive effort has been expended in the past decades to characterize the biological function of NO in biology and medicine [1–6]. Scientists have exploited various techniques and methods including spectroscopic analysis, electrochemical sensors, fluorescent dyes, electron spin resonance, or chromatographic determinations to understand the complex NO metabolism in biological samples or even whole organisms [7–16]. On account of its major importance and relevance in physiology and pathological processes, the urgent need arose to visualize NO quantitatively, directly and with high accuracy in a dynamic manner [17,18]. However, due to its low concentration, short lifetime, and extremely high reactivity with various reactive oxygen species, the direct real-time visualization of NO represents a serious challenge [17,18]. All methods mentioned above have certain individual advantages; however, all of them have also severe disadvantages that limit their application to answer specific question related to NO signaling [17,18]. Most approaches provide only a direct or indirect read-out of NO values averaged over a population of cells. Single cell imaging techniques are quite different and enable scientists to gain important insights in cell signaling events [19,20]. Moreover, single cell imaging approaches provide high-content information about the spatiotemporal patterns of molecular processes and reveal cell-to-cell variabilities [21,22]. Such highly informative readouts can be obtained by high-resolution microscopy of fluorescent probes within cells [23–25]. Thanks to the remarkable technological progress in the field of fluorescent biosensor development during the preceding years we are now able to selectively visualize NO and NO-related species in a real-time manner on the cellular or even subcellular level [20,23,25]. By employing variously colored and targeted probes in a given cell, this approach also enables measurements of different signals such as NO and Ca^{2+} dynamics simultaneously and in distinct subcellular locals [26–28]. In this review we introduce and summarize features of novel genetically encoded sensors (GES) for NO imaging which have been developed recently.

2. Genetically encoded fluorescent sensors (GES), the basic principles

GES are indispensable tools that have revolutionized and

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Table 1
Classes of genetically encoded fluorescent sensor.

Class 1 GES:
Translocation based probes, which report cell signaling events by alterations of local fluorescence accumulations (Fig. 1A.) [30,31].
Class 2 GES:
Intensity based single fluorescent protein (FP) probes, which sense dynamics of ion concentrations by changing their fluorescence intensity (Fig. 1B.) [32,33].
Class 3 GES:
Ratiometric single FP probes that sense cell signaling events by distinct changes of their spectral properties (Fig. 1C.) [34–37].
Class 4 GES:
Förster resonance energy transfer (FRET)-based probes, which are activated by conformational changes of the probe in the presence of the analyte (Fig. 1D-E.) [38,39].
Class 5 GES:
FRET-based sensors for enzyme activity or activation. These probes are modified by enzymes which affects their conformation and, hence, the FRET signal (Fig. 1E.) [40,41].
Class 6 GES:
FRET-based sensors, which are activated by enzyme cleavage *e.g.* caspase sensors (Fig. 1F.) [42,43].

accelerated biological studies [24,29]. Until now, more than 100 different GES have been developed to monitor distinct biomolecules and (sub)cellular processes including metabolites, signaling molecules, ion fluxes, and enzymatic activities with high spatial and temporal resolution [19,22,29]. Apparently, the development of novel GES and improvements of existing GES are ongoing processes [29]. Based on their operating principle, GES are usually further divided into different classes (Table 1 and Fig. 1.) [19].

Class 1 GES comprise a huge family of constructs, designed to visualize the subcellular distribution of signaling proteins by fusing it to a fluorescent protein (FP) variant (Fig. 1A.). Single cell imaging of cells expressing FP fusion constructs can provide a high-resolution readout of certain cell activities, if the FP-tagged protein significantly changes its subcellular location during specific cell signaling events [30,31]. In several studies, expression of eNOS fused to GFP or other FPs has been used to visualize the subcellular localization and translocation of the NO producing enzymes in single cells in response to diverse stimuli and stresses [26,44–47]. These experiments demonstrated that the subcellular localization of eNOS is dynamically altered. The subcellular localization of eNOS determines its enzymatic activity, which is known to be tightly regulated by protein-protein interactions, the local availability of (co)substrates, second messengers such as Ca^{2+}, and diverse posttranslational modifications of the enzyme [26,44–48]. However, the visualization of dynamic NOS-FP translocation events in single cells might, if at all, only represent an indirect readout of NO formation as NOS redistributions not necessarily correlate with its enzymatic activity.

Class 2 GES are intensity based probes. Here the fluorescence intensity of a single FP is affected by the concentration of the analyte of interest (Fig. 1B.). In its simplest form the intrinsic sensitivity of some naturally occurring or mutated FP to *e.g.* H^+ or chloride (Cl^-) is exploited to measure the dynamics of intracellular pH or Cl^- variations, respectively [32,33]. In addition, a novel class of genetically encoded NO probes, the geNOps, which will be described in detail later in this review, represent single FP quenching-based NO indicators and, hence, are rationally designed as a class 2 GES [27]. In geNOps the NO sensitivity of FPs, which naturally remain unaffected by NO, was introduced by fusing a bacteria-derived NO binding domain directly to FP variants in order to bring the radical in close vicinity to the FP chromophore [27].

Another class of single FP-based probes, referred to as class 3 GES, are ratiometric probes as their spectral properties are mutually affected by binding of the analyte of interest (Fig. 1C.). Most of these probes contain circularly permuted FPs conjugated with respective sensor domains. The Ca^{2+} sensitive pericams and GECOs as well as the hydrogenperoxide (H_2O_2) sensitive HyPer and HyPerRed are examples of class 3 GES [34–37]. Respective ratiometric single FP-based probes sensitive to NO have not been developed so far.

Many GES (class 4, 5, and 6) are based on Förster resonance energy transfer (FRET), a phenomenon that occurs when two fluorophores with overlapping excitation and emission spectra are closely aligned [38,39]. FRET-based GES naturally provide a ratiometric read-out. The FRET ratio signal may respond to the binding of an analyte (class 4, Fig. 1D-E.), posttranslational modifications (class 5, Fig. 1E.) or the cleavage of a peptide motif (class 6, Fig. 1F.). As a consequence the distance between the FRET-donor and FRET-acceptor FP and hence the FRET ratio signal is either increased or decreased [38–43]. Many FP variants are available for engineering FRET-based probes [19,24]. However, FRET-based GES most frequently consists of cyan FPs (CFPs) and yellow FP variants (YFPs) as FRET donors and FRET acceptors, respectively [38–41,43]. In addition, red-shifted FRET based probes have been developed using green FPs (GFPs) and orange (OFPs) or red FPs (RFPs), respectively [49,50]. While red-shifted FRET-based GES often have a lower dynamic range, they can be spectrally separated from UV-excitable indicators such as fura-2, a popular small chemical Ca^{2+} probe, to correlate the spatiotemporal patterns of certain cell signaling events with Ca^{2+} signals in one given cell [49,51]. For the development of direct and indirect genetic NO probes the FRET-technology has been exploited as described in chapters 3.2, 4.1 and 4.2.

3. Direct NO probes

3.1. The geNOps, differently colored FP quenching-based NO probes

We have recently developed a novel class of genetically encoded NO probes, which we named geNOps [27]. These probes consist of different FP variants directly fused to a bacteria derived NO binding domain (Fig. 2.). This domain, referred to as GAF, selectively binds NO *via* a non-heme iron(II) center (Fig. 2.). GAF is actually the NO sensitive domain of the transcription factor NorR, which is expressed in intestinal bacteria to protect them from toxic NO by inducing the expression of enzymes that convert NO to non-toxic laughing gas, the dinitrogen monoxide molecule (N_2O) [52]. However, in geNOps the NO-binding GAF domain brings the radical in close vicinity to the chromophore of FPs, thereby probably affecting the electron density resulting in an immediate loss of fluorescence (Fig. 2.) [27]. Importantly, when NO dissociates from geNOps their fluorescence is fully recovered. Accordingly, geNOps enable the real-time visualization of NO dynamics on the level of individual cells using fluorescence microscopy [27,28].

Others and we have used the differently colored geNOps to investigate NO signals in endothelial cells, vascular smooth muscle cells and HEK293 cells expressing eNOS, nNOS or iNOS under various experimental conditions [26,27,53]. The geNOps proved suitable to correlate Ca^{2+} and NO signals by combining fura-2 with either the green or orange geNOp, G-geNOp and O-geNOp, respectively [26,27]. In a recent video article we have introduced this powerful approach describing the respective protocol steps in detail [28]. Moreover, mitochondria targeted geNOps have been developed and used to visualize NO signals within this important organelle in endothelial cells [27]. Interestingly, we found a positive correlation between mitochondrial Ca^{2+} uptake and eNOS-mediated NO production exploiting the geNOps technology [26]. While the molecular mechanisms responsible for this phenomenon have not yet been discovered, these findings might lead to novel therapeutic strategies for the treatment of cardio-vascular diseases that are related to a disturbed NO homeostasis [26]. Recently, primary smooth muscle cells were infected with an AVV5 virus coding for C-geNOp in order to study the conversion of nitroglycerin to NO by the aldehydehydrogenase isoform 2 (ALDH2) and a respective ALDH2 mutant on the level of individual cells [53]. The usage of geNOps in HEK293 cells expressing different NOS isoforms unveiled that nNOS produces NO much faster and in a pulsatile manner compared to eNOS