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Review Article

Redox biosensors in a context of multiparameter imaging

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ABSTRACT

A wide variety of genetically encoded fluorescent biosensors are available to date. Some of them have already contributed significantly to our understanding of biological processes occurring at cellular and organismal levels. Using such an approach, outstanding success has been achieved in the field of redox biology. The probes allowed researchers to observe, for the first time, the dynamics of important redox parameters in vivo during embryogenesis, aging, the inflammatory response, the pathogenesis of various diseases, and many other processes. Given the differences in the readout and spectra of the probes, they can be used in multiparameter imaging in which several processes are monitored simultaneously in the cell. Intracellular processes form an extensive network of interactions. For example, redox changes are often accompanied by changes in many other biochemical reactions related to cellular metabolism and signaling. Therefore, multiparameter imaging can provide important information concerning the temporal and spatial relationship of various signaling and metabolic processes. In this review, we will describe the main types of genetically encoded biosensors, the most frequently used readout, and their use in multiplexed imaging mode.

1. Introduction

Redox reactions play a key role in almost all cellular processes. Maintaining redox homeostasis is essential for cell survival. Therefore, redox imbalance usually indicates stress or the development of pathological conditions. However, even in normally functioning cells, redox fluctuations occur regularly as a part of redox signaling pathways. In cells, redox signals are transduced via reactive oxygen species (ROS), primarily hydrogen peroxide (H_2O_2), or reactive nitrogen species (RNS) [1–7]. Very recently, ROS were considered to be toxic by-products of aerobic metabolism, leading to oxidative stress or cell death. However, it was found that low concentrations of ROS are essential for normal cell functioning [4,8,9], affecting processes such as the inflammatory response, cell proliferation, aging and development, and stem cell biology [5,6,10,11]. The main mechanism by which redox signals are transduced within a cell is the reversible oxidation of thiol groups of proteins. The formation of intra- or intermolecular disulfide bonds between cysteine residues as well as their sulfenylation and nitrosylation upon oxidation induced by ROS work as a switch for protein activity [12–16].

Thiol-disulfide exchange reactions are controlled mostly by cellular enzymatic systems [4,17–21] that depend on the redox state of the glutathione pool. Glutathione is present in the cytoplasm at high concentrations, as it is one of the main cellular antioxidants [22–24]. Thus, the ratio of oxidized to reduced forms of glutathione (GSSG/2GSH) is one of the most important cellular redox parameters determining the redox state of thiol groups of proteins. Along with glutathione, oxidized and reduced thioredoxin form another important redox couple, TrxSS/

Abbreviations: CaMKII, Ca2 + /calmodulin-dependent protein kinase II; CFP, cyan fluorescent protein; cpGFP, circularly permuted green fluorescent protein; cpFP, circularly permuted fluorescent protein; cpYFP, circularly permuted yellow fluorescent protein; CRDR, CellROX Deep Red; DTT, dithiothreitol; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; FLIM, Fluorescence lifetime imaging microscopy; FP, fluorescent protein; FPA, fluorescence polarization anisotropy; FRET, Förster resonance energy transfer; IMS, mitochondrial intermembrane space; GAF domain, cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA domain; geNOps, genetically encoded fluorescent nitric oxide probes; GFP, green fluorescent protein; Grx, glutaredoxin; G6PD, glucose-6-phosphate dehydrogenase; MnSOD, mitochondrial manganese superoxide dismutase; NOX, NADPH oxidase; Orp, oxidant receptor peroxidase; PH-domains, pleckstrin homology domains; PI, phosphoinositides; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; pnGFP, peroxynitrite sensitive green fluorescent protein; PtdIns(3, 4)P_2, phosphatidylinositol (3,4)-bisphosphate; PtdIns(3, 4,5)P_3, Phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RNS, reactive nitrogen species; roGFP, redox green fluorescent protein; roFP, redox fluorescent protein; ROS, reactive oxygen species; rxRFP, redox red fluorescent protein; rxYFP, redox yellow fluorescent protein; SERCA, sarco/endoplasmic reticulum Ca2 + -ATPase; TMRM, tetramethylrhodamine, methyl ester; Trx, thioredoxin; Tsa, thiol specific antioxidant; yEGFP3, yeast-enhanced green fluorescent protein 3; YFP, yellow fluorescent protein

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TrxSH2, involved in the cellular thiol/disulfide exchange reactions [25]. Other important cellular parameters are the redox states of the NAD (NAD^+/NADH) and NADP ($NADP^+$/NADPH) pools: both of these molecules are involved in numerous metabolic and signaling reactions. The main biological function of NAD is the modulation of energy metabolism, whereas NADPH acts as an electron donor for reductive biosynthesis, a component of the antioxidant system and a substrate of the ROS-generating enzyme – NADPH oxidase. However, growing evidence suggests that NAD, NADP and their derivatives are involved in a broader variety of cellular processes, such as calcium homeostasis, gene expression, cell death, immunological reactions, aging and carcinogenesis [26,27].

Cellular redox reactions form a complex network of interactions, affecting the course of many cellular processes. A breakthrough in studying redox processes was the development of genetically encoded biosensors based on fluorescent proteins (FPs). Currently, such indicators represent one of the most powerful tool sets for imaging cellular parameters with high temporal and spatial resolution in biological systems of any complexity [28,29]. To date, a variety of genetically encoded redox-sensors have been developed to investigate H_2O_2 [30–32], the redox state of GSSG/2GSH [33–35], TrxSS/TrxSH2 [36], NAD^+/NADH [37,38], $NADP^+$/NADPH [39,40]. Each of these types of biosensors is described in detail in the corresponding literature. However, in this review, we would like to address the subject of using redox biosensors in a different light. In living biological systems, all processes are closely interrelated, forming a complex network of interactions. Changing one parameter often leads to a change in the other. For example, oxidative stress is accompanied not only by a burst of ROS but also by changes in the redox status of the glutathione and NAD(P) pools. It is important that not only redox but also all intracellular processes are related to each other. Therefore, it is desirable to simultaneously monitor as many parameters as possible to better understand the studied phenomenon. Due to the diversity of genetically encoded biosensors, which differ in their spectral characteristics and readouts, it has become possible to simultaneously monitor several parameters in one system.

In this review, we will consider the main types of genetically encoded biosensors. To a greater extent, we will focus on redox biosensors and classify the types of their readouts and their main principles. An important chapter of this review is devoted to modern applications of genetically encoded sensors in multiparameter imaging mode.

2. Genetically encoded fluorescent biosensors: main principles

Since the discovery of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, the collection of fluorescent proteins has significantly expanded to date. Fluorescent proteins have been found in many other organisms; moreover, a large number of their synthetic versions have been obtained [41,42]. They now represent the most powerful class of techniques for research on biological processes in living cells and organisms [42–45].

Fluorescent proteins form the basis for the development of genetically encoded indicators [29,46,47]. No precise definition of the genetically encoded fluorescent indicators exists, but they can be described as artificial proteins based on at least one fluorescent protein that are capable of determining the concentration of an analyte of interest or monitoring changes in certain cellular parameters. The fluorescent protein at the core of a biosensor determines its spectral properties. In addition, biosensors can be classified according to the types of readout.

Of particular importance are genetically encoded biosensors in the field of redox biology since participants of cellular redox reactions are often short-lived and highly reactive molecules such as H_2O_2. Before the advent of advanced tools based on fluorescent proteins, synthetic fluorescent dyes were actively used to measure reactive oxygen and nitrogen species (H_2O_2, peroxynitrite ($ONOO^-$), superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and others) [48–54]. Some of the dyes continue to be used at present, particularly because of the lack of alternative approaches. Moreover, improved dyes are being regularly developed, some of which are currently a non-alternative approach for direct detection of the test component, such as in the case of hypochlorite (ClO^-) [55–57]. However, genetically encoded biosensors have a protein nature and are produced by the cellular protein synthesis machinery that underlies a number of advantages and disadvantages in comparison with chemical probes.

First, and probably most important, this technology enables the creation of transgenic organisms for in vivo research. Tissue-specific, stage-specific or inducible promoters can ensure the spatial and temporary control of probe expression, making it possible to study the dynamics of complex biological processes, such as development [58,59] and aging [58,60,61] in vivo. Some of the synthetic dyes are able to penetrate into living cells. However, in most cases it is impossible to target the agent into the cells of a living organism especially in thick layers. In addition, the procedure of dye targeting itself implies physical impacts on the object of the study which can affect physiological processes and cause undesirable artifacts.

Second, genetically encoded biosensors can be targeted to any intracellular compartment. Subcellular compartmentalization of the probe usually does not depend on the probe structure but is controlled by specific tags that can be introduced into the protein sequence [62]. Therefore, another advantage of genetically encoded biosensors over chemical dyes is that their subcellular localization is much easier to predict and control. However, it should be taken into account that not all genetically encoded biosensors function properly in all subcellular compartments. For example, in the lumen of the endoplasmic reticulum possessing a high thiol-oxidizing environment, all members of H_2O_2-sensitive HyPer family probes [32] except TriPer [63] are almost completely oxidized [64]. The indicators of the glutathione redox ratio based on roGFP and similar proteins have the same limitation [34]. Another example is the Peredox biosensor for monitoring the NAD^+/NADH ratio. Because of its extreme affinity for NADH, it cannot be used in the mitochondrial matrix where there is a high concentration of NADH [65].

Third, genetically encoded biosensors generally have greater photostability, less phototoxicity and are not prone to leaking from the cell in the course of an imaging experiment. It all makes them suitable for longer recording sessions that cannot be conducted with chemical probes. Several strategies were developed to increase excretion half-lives of chemical dyes, however, in most cases they hamper the process of loading and a direct injection becomes the only option.

On the other hand, genetically encoded biosensors are of a complex protein nature, therefore they can have many cellular partners and can undergo different post-translational modifications which can not only significantly change cellular metabolism, but also regulate the indicator functioning. For example, some designed probes did not work properly in some expression systems as they may fail to fold, lose sensitivity for the measured parameter or demonstrate pronounced cytotoxicity.

Finally, an issue commonly arises whether genetically encoded biosensors violate the natural biological processes. It seems quite possible that probes for ROS detection act like scavengers and other probes which work on the principle of small ligand binding affect the available concentration of analyte by masking it. Unfortunately, there is not any easy way to tell beforehand if the biosensor would change the metabolism of a biological object that is why control experiments have to be conducted in each particular case. For example, Balla and colleagues studied different inhibitory effects of indicators for phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) detection and the reasons which underlie them [66]. For this purpose, different pleckstrin-homology domains that bind PIP_3 were expressed in cells as GFP fusion proteins. On the basis of obtained data they revealed that interaction between sensory domains of these probes and membrane associated proteins can violate the access of natural binding partners to the latter affecting functioning