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Review

Imaging calcium and redox signals using genetically encoded fluorescent indicators

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ABSTRACT

Calcium and redox signals are presently established as essential regulators of many cellular processes. Nevertheless, we are still far from fully understanding the physiological and pathological importance of these universal second messengers. It is becoming increasingly apparent that many cellular functions are not regulated by global changes in the abundance of Ca^{2+} ions and/or reactive oxygen and nitrogen species (ROS and RNS), but by the formation of transient local micro-domains or by signaling limited to a particular cellular compartment. Therefore, it is essential to identify and quantify Ca^{2+} and redox signals in single cells with a high spatial and temporal resolution. The best tools for this purpose are the genetically encoded fluorescent indicators (GEFI). These protein sensors can be targeted into different cellular compartments, feature different colors, can be used to establish transgenic animal models, and are relatively inert to the cellular environment. Based on the chemical properties of Ca^{2+} and ROS/RNS, currently more sensors exist for the detection of Ca^{2+}- than for redox signals. Here, we shortly describe the most popular genetically encoded fluorescent Ca^{2+} and redox indicators, discuss advantages and disadvantages based on our experience, show examples of different applications, and thus provide a brief guide that will help scientists choose the right combination of Ca^{2+} and redox sensors to answer specific scientific questions.

Contents

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1. Introduction

Calcium and redox signals are presently established as indispensable determinants of many cellular functions [1–5]. Regarding the redox signals; however, only about three to four decades ago this was not a generally accepted opinion. Reactive oxygen- and nitrogen species (ROS/RNS), which are major regulators of the cellular redox status, have been considered only as toxic byproducts of cellular metabolism and protein oxidation was mostly connected with pathological processes [3,6,7]. The extensive research efforts in the past years fully reversed this dogma [3,4,8,9]. We now know that exchange of electrons between cellular components of protein and non-protein nature is as vital as the exchange of phosphorylation groups or as the spatio-temporal control of Ca^{2+} levels within a cell and its compartments. Why did we not come to this conclusion much earlier? The most obvious answer is that we did not have the tools. For comparison, we did and still know much more about the functional importance of Ca^{2+} signaling mostly because we knew how to detect and quantify Ca^{2+} much earlier than we knew how to detect ROS and RNS, and to evaluate cellular redox state. There are certainly additional reasons for this difference, but it is an undeniable fact that the discovery of small chemical fluorescent Ca^{2+} indicators such as Fura, which enabled scientists to visualize Ca^{2+} dynamics in a real time and in single cells, initiated a small revolution in the field of Ca^{2+} research [10]. Moreover, advances were achieved by the establishment of the "patch-clamp" technique which allowed scientists, for the first time, to measure ion currents across cellular membranes of single cells [11,12]. Such revolutionary developments in measuring redox signals came, unfortunately, much later and even today the field needs additional tools which will allow more precise quantification and identification of not only ROS and RNS, but also redox couples, such as reduced and oxidized glutathione (GSH/GSSG), NAD^+/NADH and $NADP^+$/NADpH, which all determine the cellular redox state and functional outcome.

The most popular approaches to visualize intracellular Ca^{2+} involve utilization of fluorescent dyes and/or protein sensors [13–17]. Similar methodologies are used to detect ROS and RNS [18–25]. One of the most popular chemical ROS sensors is the 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCFDA). H_2DCFDA probe is intensiometric, irreversible and is oxidized by several ROS. Furthermore, in contrast to the Ca^{2+} detecting dyes, this dye is also prone to artefacts [26,27]. It has to be noted that new and more reliable dyes have been developed recently [28,29]. However, these are still not displaying the popularity of the above mentioned chemicals such as H_2DCFDA. Hence, genetically encoded sensors, which have been developed only in the last 15 years, are the method of choice in measuring intracellular oxidants in single living cells [24,25]. Unfortunately, this statement applies only for one out of the more than twenty biologically important ROS and RNS, the most stable specie: hydrogen peroxide (H_2O_2). Compared to measuring Ca^{2+}, all ROS and RNS are very difficult to detect mostly due to their physico-chemical properties. While ROS and RNS are highly reactive and extremely unstable, Ca^{2+} ions are relatively inert molecules able to form complexes without changing their molecular structure in the process. The latter is an additional attribute which renders Ca^{2+} much easier to detect and quantify.

There are many excellent reviews describing the development and the properties of fluorescent dyes and genetically encoded fluorescent indicators for detecting Ca^{2+} [30–35] and redox signals [24,25]. Here, we list the most widely-used probes for detecting Ca^{2+} ions, ROS and cellular redox status. Based on our own experience, we make suggestions and discuss what needs to be considered in choosing the appropriate probe to answer a specific scientific question. Moreover, given that redox and calcium signaling are very often tightly interconnected, we provide examples on how to simultaneously detect these physiologically very important signals.

Our aim is to provide a short guide which will help scientists measure Ca^{2+} and redox signals in the same or in different cellular compartments using GEFIs.

2. Calcium sensors

The discovery and the characterization of fluorescencent proteins (FP) [36–38] opened a new research area in developing Genetically Encoded Ca^{2+} Indicators (GECIs). GECIs are based on FPs, which can change their fluorescence properties upon binding of Ca^{2+} [39]. These new tools provide some advantages compared to dyes, such as specific targeting to subcellular compartments or organelles. The progression in developing and improving GECIs allowed scientists to measure signals with a higher spatial resolution. In addition, they can also be fused to proteins enabling the observation of Ca^{2+} levels in subcellular micro-domains. These new features allow novel insights into signaling pathways and the regulation of the Ca^{2+} homeostasis.

Based on their properties, GECIs can be divided into two main groups: the FRET-type cameleon sensors [13,35,40–45] and the single FP-type sensors [13,17,35,46,47].

The first family of GECIs benefits from the Förster/Fluorescence resonance energy transfer (FRET) changes enabeled by Ca^{2+} responsive elements [48–50]. One of the first FRET-based Ca^{2+} sensors was developed by inserting the calmodulin-binding domain between blue fluorescent protein (BFP) and green fluorescent protein (GFP) [43]. After the binding of endogenous Ca^{2+}-bound calmodulin to the sensor, the distance between both proteins increases thus generating a decrease of FRET as the reporting signal. Later, cyan fluorescent protein (CFP)—yellow fluorescent protein (YFP) became the most popular FRET pair in GECIs known as yellow cameleons (YCs). In general, all YCs use a FRET pair with a donor fluorophore and a red-shifted acceptor fluorophore. Both beta-barrel fluorescence proteins are linked by Ca^{2+}-bound calmodulin (CaM) and a CaM-binding peptide [51]. Originally peptides from the skeletal muscle myosin light chain kinase (M13) or the CaM-dependent kinase (CKKp) are used as CaM binding peptides [45]. Later troponin C from chicken skeletal muscle and human cardiac muscle was introduced as a Ca^{2+}-responsive element, as for example in the TN-XL sensors [52,53]. This modification allowed reducing the sensor interference with the endogenous CaM. A Ca^{2+}-induced conformation change of the Ca^{2+}-responsive element facilitates the approach of both fluorophores allowing unimolecular FRET [51], resulting in the readout of the acceptor/donor fluorescence ratio in relation to the Ca^{2+} concentration. The ratiometric indicators exhibit small signal changes in their distinct wavelengths of the Ca^{2+}-free and Ca^{2+}-bound states but benefit from their intrinsic ratiometric signal changes [54]. The ratiometric properties render these sensors more quantitative and less susceptible to imaging artefacts [39,55,56], which can be caused by light induced bleaching, expression differences between cellular compartments and/or different cells, etc. Variants with shifted equilibrium dissociation constants for Ca^{2+} (K_d) were generated by small genetic changes to provide variants optimized for the usage in environments containing low [57] or high [58–60] concentrations of Ca^{2+}. However, due to the presence of two different FPs, the possibilities for multicolor imaging are limited [61,62]. Moreover, older versions of the sensors have potential problems through perturbations by cellular proteins, such as endogenous CaM. Newer FRET-based GECIs overcome this problem by a mutated CaM-peptide pair, which is no longer influenced by large excesses of CaM [58].

The second GECI family contains a large group of single-FP-type sensors, which are usually based on a single circularly permuted FP (cpFP) [63,64]. For the majority (GCaMPs, G-CaMPs and pericams) the Ca^{2+}-sensing CaM domain and the M13 peptide are fused to