Journal of Photochemistry and Photobiology C: Photochemistry Reviews 30 (2017) 24–35

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology C: Photochemistry Reviews

journal homepage: www.elsevier.com/locate/jphotochemrev

Invited Review

Targetable fluorescent sensors for advanced cell function analysis

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ARTICLE INFO

Article history:
Received 14 September 2016
Received in revised form 17 January 2017
Accepted 20 January 2017
Available online 29 January 2017

Keywords:
Fluorescent sensor
Probe targeting
pH sensing
Protein labeling
Fluorogenic labeling

ABSTRACT

Chemistry-based bioimaging techniques have contributed to the elucidation of intracellular physiological events. During the last few decades, many fluorescent sensors have been developed and used in live cell experiments. Owing to immense efforts by numerous research groups, several strategies have been developed to design fluorescent sensors based on various components such as small molecules and fluorescent proteins. Recently, site-specific targeting of fluorescent sensors has attracted increasing attention. Strategies for fluorescent sensor targeting were surveyed in this review with the aims to expand current knowledge on chemistry-based bioimaging and aid in the emergence of related innovative technologies. The first discussed strategy is based on the intrinsic properties of small molecules for localization at specific organelles, such as mitochondria, nuclei, and lysosomes. As a further elaboration of the topic, our recent study about in vivo targeting of pH sensors was briefly introduced. The second strategy exploits genetically encoded tags and their specific ligands. Here, fluorescent sensors with commercially available tags and corresponding ligands were mainly reviewed. As the final topic, our original protein labeling technique, which enables fluorogenic labeling as an advanced technology, was introduced.

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1. Introduction

When Roger Y. Tsien reported the first fluorescent sensor for free Ca^{2+} [1], such chemistry-based bioimaging techniques opened a new era of cell biology. Newer Ca^{2+} probes, in particular Fura-2 [2], have contributed enormously to the elucidation of intracellular Ca^{2+} signaling events [3]. The evolution of these tools essentially created a new field of biology called "chemical biology" and in the process formed a new relationship between physiologists and synthetic chemists. In the period from the 1990s to 2000s, many flu-

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http://dx.doi.org/10.1016/j.jphotochemrev.2017.01.003
1389-5567/

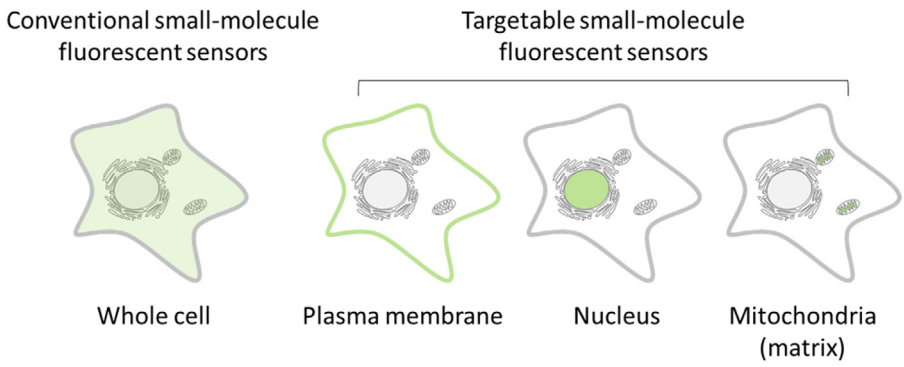

Fig. 1. Subcellular distribution of conventional fluorescent sensors and organelle targetable fluorescent sensors.

orescent sensors were developed and have subsequently been used in live cell imaging experiments [4]; in some cases, near-infrared sensors have been used in *in vivo* studies [5,6].

Soon after fluorescent proteins (FPs) became familiar tools for molecular biologists, two FP-based sensors were developed for Ca^{2+} imaging [7,8]. Both sensors took advantage of the fluorescence resonance energy transfer (FRET) phenomenon, whereby the energy of an excited donor fluorophore is non-radiatively transferred to an acceptor fluorophore [9]. These protein-based sensors are genetically encoded; therefore, they can be targeted to specific subcellular domains by linking a signal peptide sequence with the protein sensors [10–12]. In comparison, classical small-molecule sensors do not have this targeting property.

However, small-molecule sensors have several advantages over protein sensors, including an established probe design strategy [13]. For example, the rational design of a FRET-based sensor would also be effective for small-molecule sensors [14]. Photoinduced electron transfer (PeT) and internal charge transfer (ICT) have also often been utilized as other rational design strategies for small-molecule fluorescent sensors [15,16]. In addition, various physical properties of small-molecule sensors such as a large dynamic range of the fluorescence intensity, excitation/emission wavelength, or dissociation constants to the target are tunable by the organic synthesis-based molecular design. Some organic fluorophores such as ATTO647 are much more photo-stable than FPs, and are thus applicable to strong laser-based imaging such as STED (stimulated emission depletion) microscopy [17]. Therefore, small-molecule fluorescent sensors are still useful for a variety of biological applications, and thus it is of importance to develop advanced functional, target-specific small-molecule fluorescent sensors to address these needs (Fig. 1).

Strategies to develop target-specific small-molecule sensors can be classified simply into two approaches. One utilizes the intrinsic properties of fluorophores or other small molecules for targeting specific regions such as subcellular organelles. The second exploits the combination of a genetically encoded tag and its specific ligand. In this review, I will briefly discuss these two probe targeting strategies and then introduce our recent studies in this area.

2. Probe targeting strategy based on the intrinsic properties of small molecules

2.1. Targetable fluorescent sensors based on the intrinsic properties of small molecules

In the simplest case, a fluorophore may possess the property of being able to be targeted to a subcellular location. Over the last several decades, various organelle-specific fluorescent probes have been reported [18]. Fluorescent probes such as DAPI (**1**), MitoTracker (**2**), LysoTracker (**3**), ER-Tracker (**4**), and their derivatives (Fig. 2) are commercially available and have been widely used for targeting nuclei, mitochondria, lysosomes, and the endoplasmic reticulum, respectively. An examination of the structure of MitoTracker Orange revealed that this compound is a derivative of rhodamine, somewhat like tetramethylrhodamine, which is one of the most popular fluorophores in biology. Rhodamines have a carboxy group at the *ortho*-position of the 9-substituted phenyl group; thus, they exist as neutral intramolecular salts. When the carboxylate anion is removed or modified to an ester, the total charge on the molecule become positive. In these cases, the fluorophores tend to accumulate within mitochondria. Cyanine family or other cationic fluorophores are also known to accumulate in mitochondria [19,20]. By utilizing the nature of the fluorophores, rhodamine-based sensors for Ca^{2+} (**5a**) as well as Zn^{2+} (**5b**) and cyanine-based pH sensors (**6**) have been reported to be mitochondria-targetable fluorescent sensors (Fig. 3) [21–24]. Hamachi et al. also developed a pyronine-based nucleotide sensor (**7**) for targeting mitochondria [25].

Mitochondria have two lipid membranes, an outer membrane and an inner membrane. Unlike the outer membrane, the inner membrane is highly impermeant to molecules. Numerous transporters and ion channels are therefore required to allow molecules to move in and out of the mitochondrial matrix. As a result, the mitochondrial inner membrane has a membrane potential that is maintained at about −180 mV. As a distinguishing feature of this organelle, a "lipophilic cation" has become a common structural feature for targeting molecules to the mitochondria. Fluorophores that have a tendency to accumulate in mitochondria, such as some rhodamine derivatives, generally meet this criterion. However, a more reliable method for organelle targeting is through conjugation with an organelle-targeting small molecule, despite the fact that this method increases the molecular weight. The most popular mitochondria-targeting agents are triphenylphosphonium (TPP) salts [26,27]. This strategy has proven to be very useful in the molecular design of mitochondria-targeting sensors for H_2O_2 [28], Zn^{2+} (**8**) [29], Cu^{+} (**9**) [30], Mg^{2+} (**10**) [31], and H_2S (**11**) [32] (Fig. 4).

The lysosome is one of the most important digestive organelles in cells, where various macromolecules are degraded. The pH in lysosomes is maintained in the acidic range (pH 4.5–5.0) due to the action of a series of proton pumps. Lysosomes are surrounded by a lipid membrane; thus, lysosome-targeting compounds need to exist in part in the non-charged form at neutral pH. Tertiary amines are widely used lysosome-targeting groups, such as an *N*,*N*,-dimethylaminoethyl group and a morpholinoethyl group (Fig. 5). At cytosolic neutral pH, these amino groups are partially protonated, so that their neutral portions can permeate into the lysosomes. Once entering acidic compartments, these functional groups are almost fully protonated, and thus the hydrophilicity of the total compounds increases and the compounds become trapped in the compartments. Since visualization of the metal ions in the lysosome