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INVITED REVIEW

Small-molecule fluorophores and fluorescent probes for bioimaging

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Abstract Fluorescent compounds based on synthetic small molecules are powerful tools to visualize biological events in living cells and organisms. Ever since the discovery of organic fluorescent compounds in the late nineteenth century, efforts have been made to "see" the behaviors of specific biomolecules in living systems by using these dyes as labels. Also, following the development of fluorescent Ca^{2+} indicators in the 1980s, many fluorescent probes or biosensors, which are defined as molecules that show a change in fluorescence properties in the presence of their target molecule, have been reported and applied in biological research. Today, a variety of probes are available that target metal ions, pH, enzyme activities, and signaling molecules. In this review, we first consider the history of organic fluorescent molecules and discuss their utility for labeling biomolecules and staining cells. Then, we review recent progress in small-molecule fluorescent probes for metal ions and reactive oxygen species, focusing on representative work in each category. Finally, we briefly discuss attempts to create novel kinds of probes, including hybrids of small molecules and genetically encoded proteins, with the potential to overcome some of the limitations of current probes.

Keywords Fluorescence · Imaging · Ca^{2+} signaling · Reactive oxygen species · Protein labeling

Introduction

It is said that humans rely on vision for approximately 80 % of all the information they receive from the external world. Hence, as the proverb "seeing is believing" suggests, an appeal to the eye should be most effective and informative even in the world of science. Unfortunately, however, our body and cells generally do not supply enough visual information detectable with the naked eye to allow us to understand specific biological events, such as cell signaling and protein expression. Therefore, tools that allow us to "see into the body" or "see into cells" are essential not only for basic biology research, but also for the diagnosis and treatment of diseases. Suitable methods must meet several conditions. First, the technique should be sensitive enough to visualize biological compounds at physiological concentrations, which in most cases range from nanomolars to micromolars. Second, the method should have sufficient spatial and temporal resolution to analyze dynamic cell signaling processes. Third, the method should not be invasive, i.e., detection should be possible from outside of the samples. Last, but not the least, it should rely on instruments that are not too special or expensive. At least for cells and dissected tissues, fluorescence imaging is an ideal methodology that satisfies all these requirements, compared with other technologies based on radioactivity, bioluminescence, electromagnetism, and electrochemistry. This is the reason why fluorescence live imaging is coming into widespread use in cell biology. Another advantage of the technology that we should emphasize here is that the fluorescence signal of a molecule can be drastically modulated by design, so probes that exhibit *activation* of the signal, not just *accumulation* of the compound, can be exploited, as we will describe later.

Needless to say, to perform fluorescence imaging, fluorescent molecules must be present in the sample. Although some researchers have used internal or endogenous fluorescent molecules in cells [11,12,38,122,133], including tryptophan, NAD(P)H, and flavins, in most cases, external fluorophores are added to the samples, either chemically or genetically. Before the middle of the 1990s, when fusion of green fluorescent protein [131] was introduced as a labeling

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tool for proteins in general [19,145], organic fluorescent molecules were the only choices for fluorophores. Although fluorescent proteins are currently widely used, small-molecule fluorescent compounds are still important as both labeling agents and activatable sensors because (1) they are applicable to any sample, including the human body, (2) they are relatively inexpensive and easier to handle, and (3) they can generally provide high signal-to-noise ratios (up to over 1,000) as a result of ingenious chemical design (vide infra).

Therefore, in this review, we focus on organic small-molecule fluorophores and their use as labels and sensors for bioimaging. Probes using fluorescent proteins [26,109,165], quantum dots [98,134], lanthanide ions [13,90,138], and other new platforms are outside the scope of this review. Also, due to space limitation, we describe only representative small-molecule probes here. Various monographs and review articles provide detailed coverage of fluorescence spectroscopy [58,83], fluorophores [47,51,96,97], and fluorescent probes [63,64,77,139,160] for readers who require further information.

Small-molecule fluorophores

Although phenomena ascribed to fluorescence had been observed for water infusion from a plant [111] and for some kinds of rock in the early modern period, the first fluorescent organic molecule to be specifically identified was quinine sulfate (Fig. 1a), which was reported by Sir John Herschel in 1845 [54]. Strictly speaking, this is not quite correct because the word "fluorescence" was not coined until 1852, when George Stokes published a long paper on the subject [136]. Since the discovery of quinine sulfate, which emits blue fluorescence when irradiated with ultraviolet (UV) light, many other organic compounds have been found or created to fluoresce in a variety of colors. Fluorescein (Fig. 1b) [4] and rhodamine (Fig. 1c) [18] derivatives, which were first reported as early as the late nineteenth century, are especially important, as they are among the representative platforms currently employed in fluorescent labels and probes used for bioimaging. BODIPY dyes (Fig. 1d) [96] and cyanine dyes (Fig. 1e) [97] are also often used for this purpose.

Despite this long history, structural modifications of organic fluorescent dyes still remain to be explored. For example, fluorescein derivatives in which the carboxyl group is replaced with other functional groups, termed TokyoGreens (TGs), were not created until the twenty-first century (Fig. 1b) [149]. Bright and photostable dyes with red to near-infrared (NIR) fluorescence are especially in demand for in vivo imaging in animals and humans, owing to the transparency of tissues in this wavelength region [97,157]. This issue has been addressed by the development of new dyes with additional ring systems [147,163] and by adding heteroatoms [36,79] to existing fluorophores, as well as by the modification of cyanine dyes [127]. For example, our group has replaced the oxygen atom of TGs with a

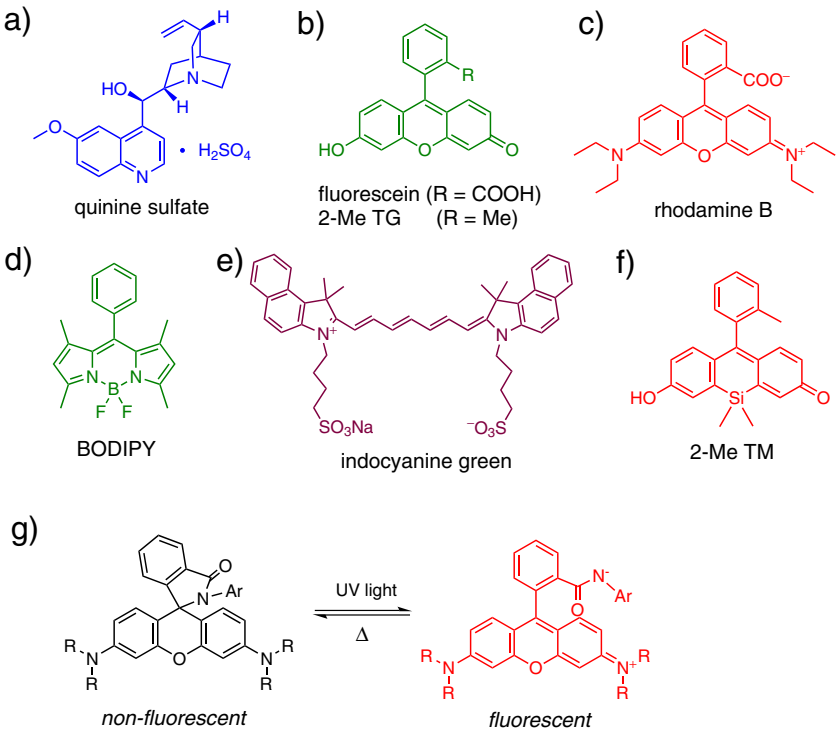

Fig. 1 Chemical structures of representative organic fluorescent molecules. **a** Quinine sulfate, **b** fluorescein and 2-Me TG [149], **c** rhodamine B, **d** 1,3,5,7-tetramethyl-8-phenyl-BODIPY, **e** indocyanine green, **f** 2-Me TM [36], and **g** a photochromic rhodamine [41]. The color of the molecules roughly represents the emission color

dimethylsilyl group to obtain novel fluorescent molecules with red emission, TokyoMagentas (TMs; Fig. 1f) [36]. Compared to fluorescein, 2-Me TM has absorption and emission maxima at around 90 nm longer wavelength. Interestingly, the dye exhibited a large hypsochromic shift of the absorption spectrum upon protonation at weakly acidic pH. Another class of fluorescent dyes that has recently attracted attention is those suitable for two-photon imaging [73,82,99] and for super-resolution imaging. Super-resolution imaging is a collective term that refers to methodologies used to overcome the diffraction limit of conventional light microscopy [59]. Fluorescent molecules that undergo reversible photoswitching [27,41,53] or irreversible photoactivation [8,89] are required for some of these imaging technologies, including stochastic optical reconstruction microscopy (STORM) [125] and photoactivated localization microscopy (PALM) [10]. For instance, Stefan Hell and others reported a photochromic rhodamine derivative whose fluorescence is turned on after UV irradiation and turned off again by thermal relaxation (Fig. 1g) [41].

Due to the difficulty of predicting the photophysical properties of molecules under physiological conditions a priori, most of the novel fluorescent molecules were discovered serendipitously [4,18] or by screening multiple compounds [75,127]. With the aim of accelerating rational and efficient development, many efforts to explain [86,115,123] and predict [87] the optical properties of fluorescent molecules by quantum calculation have been made in recent years, with partial success. It may eventually be possible to combine organic and computational chemistry to obtain new fluorescent scaffolds with superior properties to classical ones.

Fluorescence imaging with labeled molecules and staining agents

A few decades after the initial development of organic fluorescent molecules, fluorescence microscopy was invented and applied to biological research. Fluorescence microscopes were first reported by Reichert and Heimstädt in 1911 and by Lehmann in 1913, as an outgrowth of Köhler's UV microscope [37]. They used a carbon arc with appropriate filters as a source of UV light and observed the transmitted fluorescence. The first biological samples to be observed included plants, bacteria, and animal tissues that spontaneously fluoresce. Meanwhile, von Prowazek studied the binding of dyes to cells in order to make them fluorescent in 1914 [152]. This should be regarded as the first attempt to achieve fluorescence staining of cells. Another important development was made in 1942 by Coons, who covalently labeled antibodies with fluorescein isocyanate and used them for tissue imaging with a fluorescence microscope [28]. This was the beginning of immunofluorescence, which is still widely used today.

Since those early works, a wide range of fluorescent labeling agents and cell staining agents has been developed and applied in biological and medical studies. For example, fluorescent dyes that selectively localize and stain a cellular target organelle, such as mitochondria, lysosomes, endoplasmic reticulum, or Golgi apparatus, are now commercially available [63]. Perhaps the most widely used are dyes for nuclear staining, such as 4′,6-diamidino-2-phenylindole (DAPI) [67] and Hoechst 33258 [85], which emit strong fluorescence when bound to DNA. More recent examples of organelle-targeted dyes include a series of styryl fluorophores reported by Young-Tae Chang [124] and a mitochondrial surface-specific fluorescent molecule reported by Motonari Uesugi [68]. Notably, these dyes were developed by combinatorial approaches as described in the succeeding sections.

In the last decade, in vivo imaging of tumors and other lesions with antibodies [6,117] and peptides [21,69] labeled with fluorescent dyes has attracted attention. For these applications, our laboratory has developed Si-substituted rhodamine-based NIR fluorescent labeling agents, including SiR700 [78]. When used to label tumor-specific antibodies, they are superior to conventional cyanine-based agents in terms of photostability. As shown in Fig. 2, we demonstrated

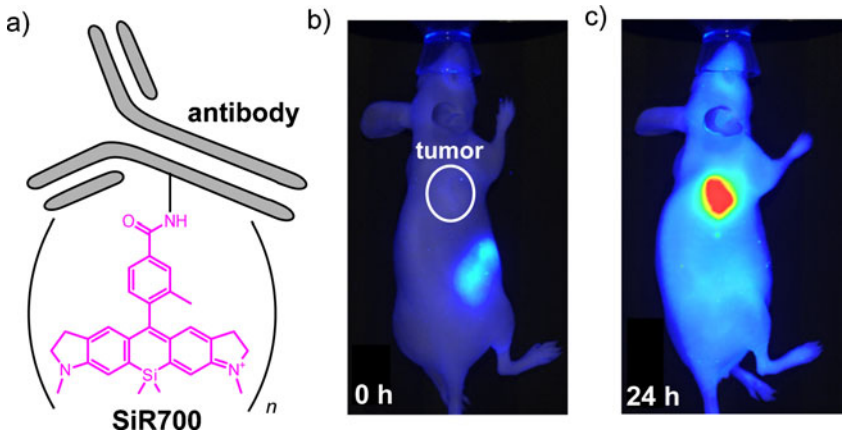

Fig. 2 In vivo tumor imaging with SiR700-labeled anti-tenascin-C antibody [78]. **a** Representative illustration of the imaging agent. **b** Fluorescence image of a mouse xenograft tumor model (prepared with HKBMM cells) before antibody injection. The *white circle* indicates the position of the implanted tumor. **c** Image of the same mouse 24 h after antibody injection. The images were taken on a Pearl Impulse Imager with a 700-nm channel

that such a dye–antibody conjugate could clearly visualize mouse tumors in vivo. Other remarkable recent reports on fluorescence imaging of specific tissues in vivo include nerve detection by Quyen Nguyen [159] and artery labeling by Prakash Kara [128].

Activatable fluorescent probes

As mentioned earlier, one of the advantages of fluorescence imaging is that the signal of a molecule can be drastically modulated. In this review, those molecules whose own optical properties are altered in response to the target chemical species or environments are defined as "fluorescent probes" (Fig. 3a). There are several rationales for the design of fluorescent probes, including photoinduced electron transfer (PeT) [16,146,149], intramolecular (or internal) charge transfer [31], Föster (or fluorescence) resonance energy transfer (FRET) [42,109], and intramolecular spirocyclization [24,65]. Schematic representations of PeT-based and FRET-based probes are shown in Fig. 3b, c, respectively. PeT is the transfer of an electron to (or from) the fluorophore at the excited state from (or to) an electron donor (or acceptor) moiety that is introduced near the fluorophore. If this process occurs, the excited state of the fluorescent molecule is quenched, resulting in low fluorescence intensity. In the case of activatable fluorescent probes, PeT is cancelled by chemical reaction of the probe with the target molecule, with consequent recovery of strong fluorescence (Fig. 3b). The kinetics and thermodynamics of PeT were formulated by Rudolph Marcus [101] and Dieter Rehm and Albert Weller [121], respectively. Early work on PeT-based fluorescent probes included the pH probes developed by A. Prasanna de Silva and others [31]. On the other hand, FRET is a nonradiative transfer of energy between two fluorescent molecules, one being the donor and the other being the acceptor. If this occurs, fluorescence of the donor molecule is weakened, and instead, fluorescence from the acceptor molecule is observed even though the donor is irradiated. For efficient FRET, the spectral overlap, as well as the distance, between the donor and acceptor chromophore is important [109]. When, for example, the linker between the two fluorophores is cleaved by a proteolytic enzyme, FRET is cancelled and the donor fluorescence is restored (Fig. 3c). In contrast to PeT-based probes, which only show a change of fluorescence intensity, FRET-based probes show a change in the shape of the excitation/emission spectra in response to the target molecule. As is well known, this feature allows for ratiometric measurement, which is highly advantageous for precise quantification of the analyte. Representative FRET-based small-molecule probes include a β-lactamase probe developed by Roger Tsien's group [166].

Because the number as well as the scope of fluorescent probes based on small molecules is continuously expanding, it is not possible to cover all of them in this short article. Instead, we will briefly describe probes for the following two categories: metal ions and reactive oxygen (nitrogen) species. Some important areas that are not reviewed herein include slow and fast membrane potential probes [39,48,49,106,153], enzyme activity probes [7,120,126], and pH indicators [112,148,158].

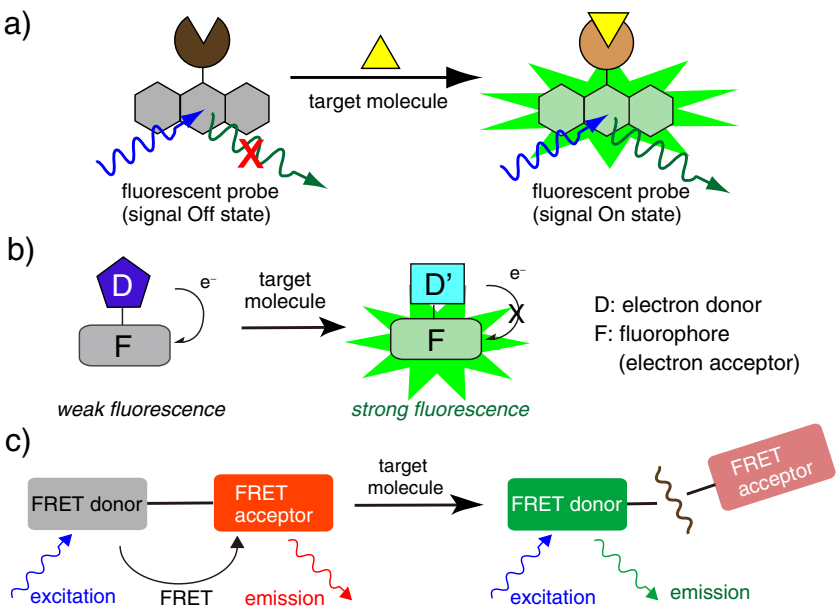

Fig. 3 Development of fluorescent probes. **a** Schematic representation of fluorescent probes, **b** design of probes based on PeT, and **c** design of probes based on FRET. For details, see the references mentioned in the text

Probes for metal ions

Probes for Ca^{2+}

The classical, and still probably the most common, target of fluorescent probes is the Ca^{2+} ion. Needless to say, Ca^{2+} as a second messenger plays pivotal roles in many types of cells, including neurons [15], eggs [34], and myocytes [9]. In 1980s, Roger Tsien and coworkers first reported a series of selective fluorescent probes for Ca^{2+}, including quin2 [144], fura-2 [50], fluo-3 [107], and rhod-2 [107] (Fig. 4a, b). These epoch-making probes demonstrated the power of fluorescence live cell imaging to biologists in every field. Even today, some of those dyes are still used to provide novel scientific insights. Importantly, the probes, together with the newer ones (vide infra), share the same chelator moiety called 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). BAPTA was developed by Tsien as a novel chelator with improved properties over the previously known ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Fig 4c) [144]. Although EGTA itself had high selectivity for Ca^{2+} over Mg^{2+}, only a small fraction of EGTA exists in an unprotonated state at physiological pH due to the high pK_a of the amine moieties (8.96 and 9.58). To overcome this problem, Tsien improved the chelator by replacing the methylene groups connecting nitrogen and oxygen of EGTA with aromatic rings. This modification lowered the pK_a values of the BAPTA amines to 6.36 and 5.47 and allowed Ca^{2+} to be well-chelated in the physiological pH range. Also, it made the sensor relatively insensitive to small fluctuations in pH.

After 1990, novel fluorescent probes for Ca^{2+} such as fluo-4 [44], Oregon Green 488 BAPTA-1 [95], and a two-photon probe [74] were developed and applied for imaging cells and tissues. For more information, see other references [63,137]. In 2011, two NIR fluorescent probes with large activation ratios in the presence of Ca^{2+} were reported independently by our group [35] (Fig. 4d) and Koji Suzuki's group [104]. These NIR probes should be useful not only for monitoring Ca^{2+} fluctuation in tissues, but also for multicolor imaging in combination with visible-range fluorescent probes.

Probes for K^+ and Na^+

K^+ is the most abundant metal ion inside cells and plays essential roles in many physiological events, such as cardiac and neuronal excitability, cellular ionic homeostasis, and cell proliferation [130]. The first fluorescent probe for K^+, potassium-binding benzofuran isophthalate (PBFI) (Fig. 4e), incorporating an 18-crown-6 base chelator, was reported by Tsien during the course of studies aimed at a Na^+-selective probe [108]. Although PBFI is commercially available [63], its biological applications are relatively limited, in contrast to the case of the Ca^{2+} probes, due to the low fluorescence quantum yield and poor selectivity for K^+ over Na^+. To address this problem, a novel K^+-selective cryptand-based chelator, 2-triazacryptand[2,2,3]-1-(2-methoxyethoxy)benzene (TAC), was recently synthesized by Huarui He [52], and several fluorescent probes using TAC as a recognition moiety have been reported [57,114,119], including TAC-Red by Alan Verkman (Fig. 4f) [119]. These probes possess improved selectivity for K^+ and can monitor changes of K^+ concentration outside the cell, where Na^+ is present in excess.

Na^+ is the most abundant metal ion in extracellular fluids. Flux of Na^+ into cells induces membrane depolarization, and abnormality of Na^+ channels is associated with human diseases [5,162]. As mentioned previously, the first fluorescent probe for Na^+ in living cells, sodium-binding benzofuran isophthalate (SBFI), was reported by Tsien in 1989 [108]. Although it is relatively selective for Na^+ and is suitable for ratiometric imaging, its excitation wavelengths are in the UV

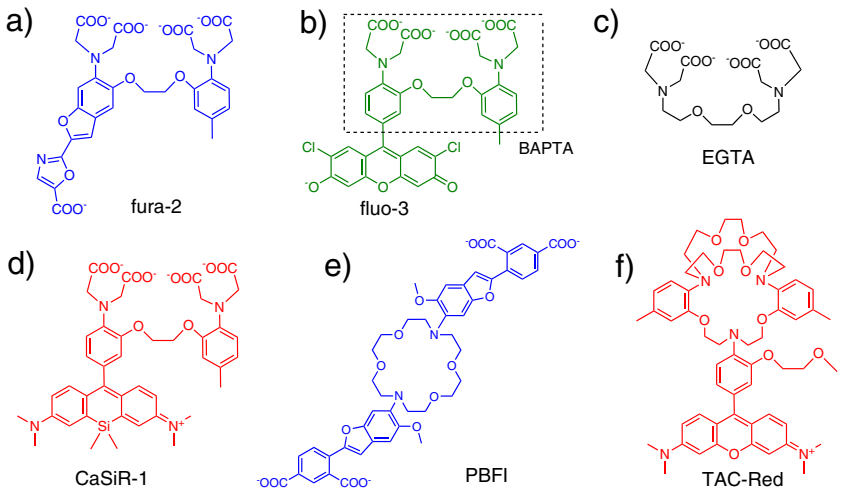

Fig. 4 Chemical structures of fluorescent probes and chelators for metal ions: **a** fura-2 [50], **b** fluo-3 [107], **c** EGTA, **d** CaSiR-1 [35], **e** PBFI [108], and **f** TAC-Red [119]

region and its brightness is limited. As improved versions, CoroNa Green [105] and CoroNa Red [63] with visible excitation wavelengths have been developed and commercialized. However, CoroNa Green has the drawback of fast leakage from live cells [105]. Very recently, Christophe Lamy solved this problem by encapsulating the probe in a dendrimer to prolong its intracellular retention while retaining the response to Na^+ [84]. Though the conjugate has to be delivered into cells by microinjection or electroporation due to its membrane impermeability, improved retention will be beneficial for imaging experiments.

Probes for other metal ions

Many metal ions other than Ca^{2+}, Na^+, and K^+ can currently be visualized by the use of specific fluorescent probes [24,33,143]. Among them, perhaps the most popular target is Zn^{2+}. Zinc is an essential metal for life and is the second most abundant transition metal in humans. The biological functions of Zn^{2+} have been well-established for the protein-bound form, but the functions of free or loosely bound (labile, mobile) Zn^{2+} are less clear [72]. In order to address this issue, many fluorescent probes for Zn^{2+} have been developed, including 6-methoxy-(8-*p*-toluenesulfonamido) quinoline (TSQ) [43], zinpyrs (reported by Stephen Lippard's group) [14], and ZnAFs (reported from our group) [56]. These probes have been reviewed in detail elsewhere [62,72,116].

Copper is also an essential metal for life, mainly due to its redox activity, and it is a popular target of fluorescent probes [24,33]. It is known that the brain needs quite high levels of copper, but at the same time, mishandling of neuronal copper stores is associated with various pathological conditions, including Menkes and Wilson's diseases [17]. In 2011, Christopher Chang developed a novel BODIPY-based fluorescent probe for Cu^+ and discovered that pools of copper in neurons undergo a dynamic redistribution that is dependent on calcium signaling [32].

Probes for reactive oxygen (nitrogen) species

Reactive oxygen species (ROS) is a collective term for activated oxygen species, including superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and others (Fig. 5a). They are important signaling molecules, which regulate a wide range of physiological functions. However, overproduction of ROS results in oxidative stress and is involved in the pathogenesis of many diseases [29]. To analyze the dynamic functions of ROS in living systems in situ, many fluorescent probes based on small molecules have been developed. The classical probes are chemically reduced forms of fluorescent dyes, such as 2′,7′-dichlorodihydrofluorescein (DCFH; Fig. 5b) [25] and dihydrorhodamine 123 [81], which are oxidized by ROS to afford strongly fluorescent products. Although they are widely used in cell imaging, auto-oxidation and lack of specificity among ROS are major drawbacks. To address these problems, probes that fluoresce only in the presence of specific ROS have recently been developed, some of which are described in the succeeding section. More comprehensive reviews of previously reported ROS probes are available [45,113,156].

Reactive chemical species analogous to ROS but containing nitrogen are known as reactive nitrogen species (RNS; Fig. 5a). The most representative RNS is nitric oxide, which is involved in many physiological and pathological processes [76]. In the last two decades, many fluorescent probes for RNS have been developed by us and others [46,91,113], but they will not be reviewed here.

Probes for hydrogen peroxide

H_2O_2 is a relatively mild ROS and has attracted intense interest recently because of its involvement in signal transduction by reversible oxidation of proteins, in addition to its established cytotoxic effects [150]. Most of the reported fluorescent probes for H_2O_2 have esters of aryl boronates as the recognition moieties; these are converted to phenols by H_2O_2 with a concomitant increase of fluorescence intensity. To our knowledge, this type of probe was first developed by Lee-Chiang Lo in 2003 [93] and vigorously improved by Christopher Chang and others [92]. A representative example, PF1, is shown in Fig. 5c [20]. Probes based on other chemistries, including ROS-mediated cleavage of benzenesulfonate groups, have been developed as well [100]. Recently, our group reported a novel probe incorporating benzil as a reactive moiety, NBzF [1]. Because benzil has a high reduction potential, the fluorescence of the probe was significantly quenched by PeT from the fluorophore to the benzil moiety, and the PeT was cancelled selectively and rapidly by reaction with H_2O_2 (Fig. 5d). These specific probes are expected to be useful in understanding the roles of H_2O_2 in living systems.

Probes for hypochlorous acid

Hypochlorous acid (HOCl) is a powerful oxidant that is produced by myeloperoxidase from H_2O_2 and Cl^- in certain types of cells, including activated phagocytes. Under physiological conditions, approximately half of HOCl is present as the hypochlorite anion (OCl^-) form. It is involved in killing microorganisms and also in many brain diseases [161]. The first practical fluorescent probe for HOCl, HySOx, was developed by our group in 2007 [70], using rhodamine spirocyclization as the rationale (Fig. 5e). With this probe, we could visualize the generation of HOCl in

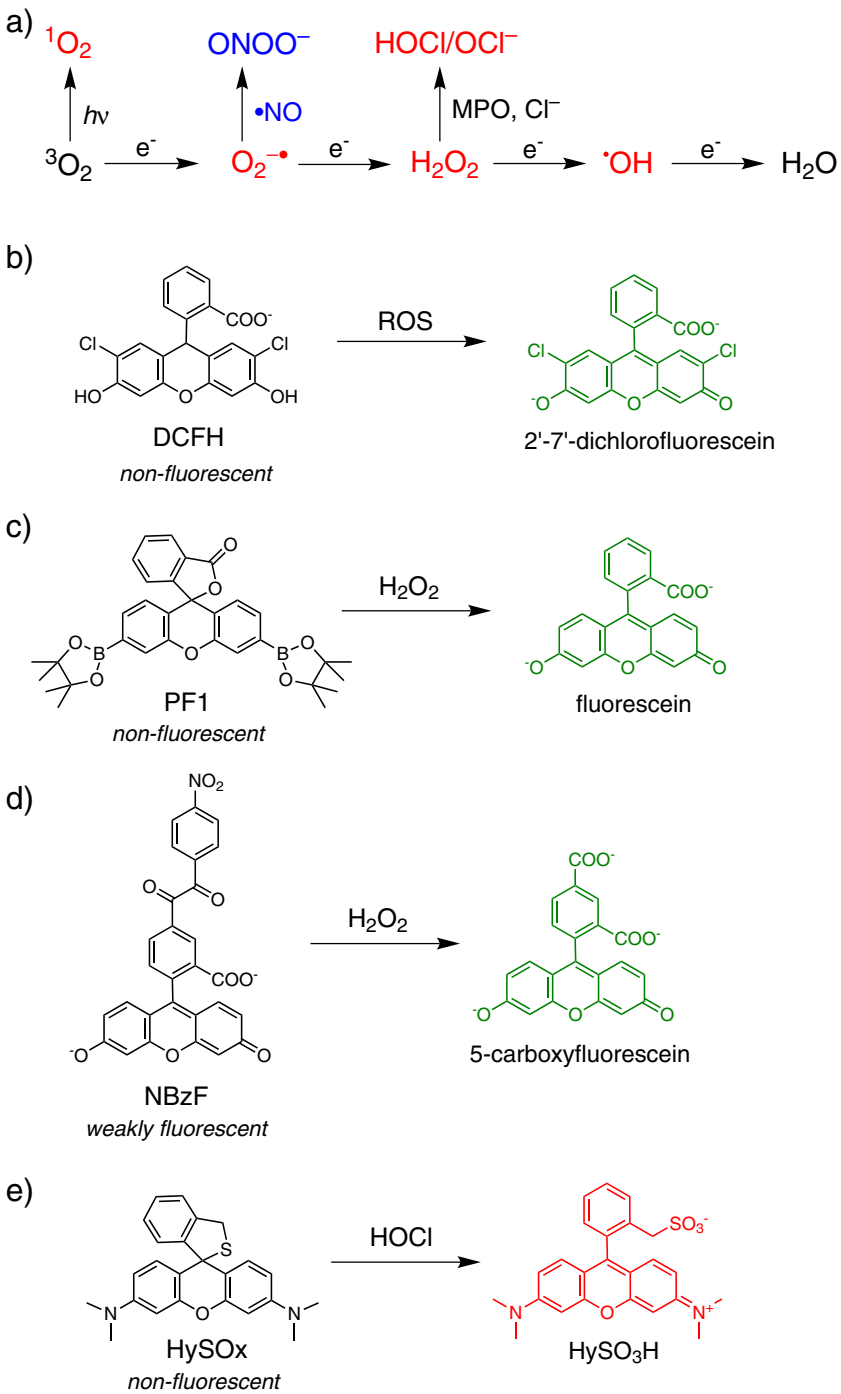

Fig. 5 ROS and fluorescent probes used to detect them. **a** Schematic relationship of various ROS (*red*) and RNS (*blue*). Chemical structures and reactions of **b** DCFH [25], **c** PF1 [20], **d** NBzF [1], and **e** HySOx [70]

phagosomes in real time. Following this work, many specific probes for HOCl have been reported based on different chemical reactions [22,23,129] and applied for bioimaging.

Novel approaches for the future

In the last part of this review, we briefly describe two new approaches that can potentially overcome the limitations of current small molecule-based fluorescent probes: combinatorial probe development and the use of protein/peptide tag technology.

Combinatorial development of probes

As described previously, small molecule-based fluorescent probes can be developed rationally by incorporating specific reactive moieties into fluorescent molecules. However, in

some cases, it is hardly possible to design a suitable chemical reaction or a recognition moiety for the target. For example, if one wants to detect cells of a specific kind, it would be very difficult to decide what target to select. To overcome this limitation, a combinatorial or diversity-oriented approach is gaining increasing attention [68,124,151]. The power of this strategy was fully demonstrated in a paper by Young-Tae Chang and others, who screened a rosamine library they had previously reported [2] and found a fluorescent compound that selectively stains pluripotent stem cells [60]. His group also prepared a BODIPY library and obtained fluorescent probes for dopamine [88] and fructose [164]. A similar approach was reported by Seung Bum Park and others [75]; they synthesized a library of compounds and screened them to find novel fluorescent molecules with excellent photophysical properties. The works on fluorogenic enzyme substrates by Jean-Louis Reymond [154] and Dalibor Sames [40] may also be classified into this category.

Use of protein/peptide tags

Although many small-molecule fluorescent probes provide a good activation ratio with high target specificity in living cells, undesired localization and retention properties of the probe often become major concerns. For example, some fluorescein-based probes are readily removed from cells [61,105], while rhodamine-based probes tend to localize in mitochondria [80]. Chemical modifications of probes, such as conjugation of chelators [61] or targeting moieties [3], are applicable in some cases, but the need for more general methodologies to control probe localization at will is clear. To address this issue, the use of tag technologies in combination with small-molecule fluorescent probes is becoming popular. Here, a tag technology corresponds to a method to couple small functional molecules (fluorescent dyes, in most cases) to genetically encoded proteins/peptides that are designed to specifically bind, either covalently or noncovalently, to molecules with characteristic chemical motifs. So far, a few tag technologies have been established, including FlAsH/ReAsH system [103], SNAP-tag [71], and HaloTag [94]. In addition, several new technologies are being developed [55,110,118].

A representative combination probe was developed by Tsien's group in 2007; they created a fluorescent Ca^{2+} probe with a biarsenical moiety that selectively binds to tetracysteine-tagged membrane proteins (Fig. 6a) and used it to monitor local Ca^{2+} dynamics [141]. Similar strategies have been reported by other groups to direct Ca^{2+} probes [66,102], a Zn^{2+} probe [140] (Fig. 6b), and a H_2O_2 probe [135] to selected organelles in order to measure local changes of the analyte.

Moreover, the scope for combining proteins with small-molecule fluorophores is not limited to these areas. For example, researchers have used solvent-sensitive (alternatively called environment-sensitive) fluorophores to monitor enzyme activities [132] or conformational changes [142] of proteins or peptides, and this approach has also been applied to monitor ion concentration [155], though these applications are beyond the scope of the present review.

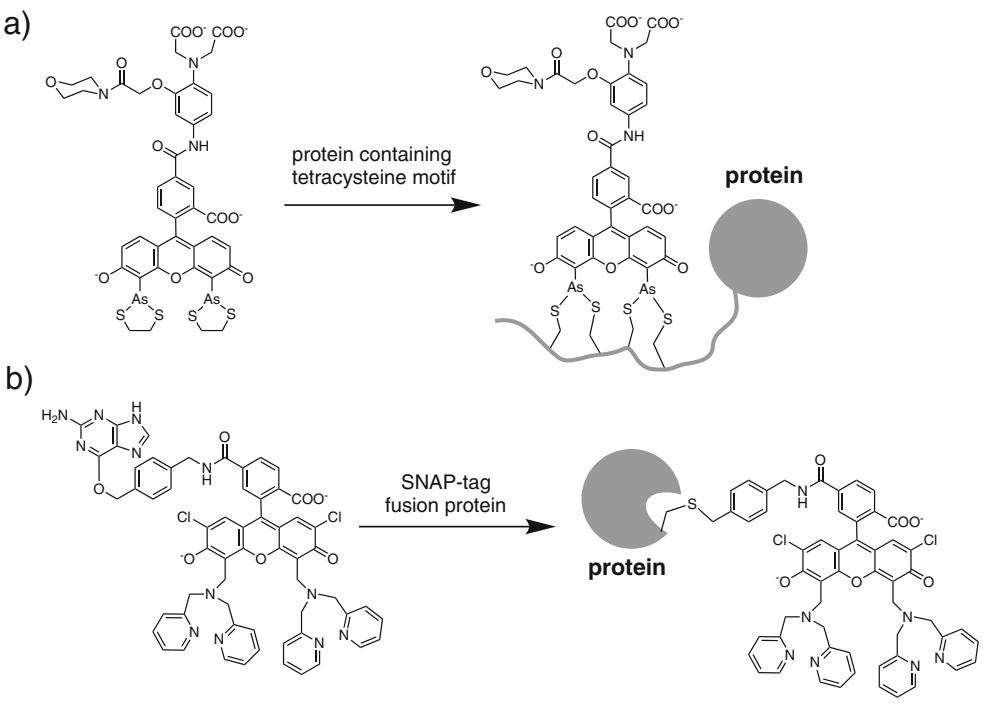

Fig. 6 Fluorescent probes with labeling moieties directed to specific tags. **a** A biarsenical Ca^{2+} probe that binds to tetracysteine-conjugated proteins [141]. **b** A Zn^{2+} probe that labels SNAP-tag fusion proteins [140]

Concluding remarks

It has been about 150 years since the discovery of organic small-molecule fluorophores. Throughout this time, numerous fluorescent molecules have been synthesized and applied in imaging of biomolecules, cells, and organisms. Introduction of novel imaging platforms, including in vivo NIR imaging, super-resolution imaging, and multiphoton imaging, has always been accompanied by the development/discovery of appropriate organic fluorescent labeling dyes that play an essential role in enabling the technologies. Importantly, the fluorescence properties of organic molecules can be rationally regulated by means of several mechanisms, allowing chemists to develop activatable fluorescent probes. At present, many fluorescent probes are available, directed to a range of targets. Without doubt, they have made significant contributions to our understanding of dynamic and complicated processes in biological systems. As mentioned previously, recently developed novel approaches should complement the conventional methodology for probes based on small-molecule fluorophores. These and other innovations are expected to spur the development of biological sciences and medicine.

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